Diet and Endothelial Function in Healthy Young People

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Dissertation submitted to University College London for the degree of Doctor of Philosophy (PhD)

March 2013

I, Julie Lanigan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm this has been acknowledged in the thesis.

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Abstract

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Cardiovascular disease (CVD) is the most common cause of death globally; yet, its pathogenesis and early development are only partially understood. Atherosclerosis begins early in life and is strongly associated with CVD risk factors including diet. Certain dietary patterns and components in particular *n-3* fatty acids have been shown to protect against CVD. However, the mechanisms through which diet operates are largely unknown.

The aim of this PhD was to investigate associations between habitual diet and the development of atherosclerosis. Participants in a randomised controlled trial investigating effects of supplementation with DHA – an omega 3 fatty acid – provided a convenience sample for this epidemiological dietary study. The main RCT outcome measure was brachial artery endothelial function measured noninvasively using vascular ultrasound (FMD). Secondary outcomes were conventional CVD risk factors including blood pressure and serum lipid concentrations.

A generalised food frequency questionnaire (FFQ) targeting all food groups was used to assess the whole diet, and from this, data were used to derive dietary patterns. Relationships between dietary patterns, FMD and CVD risk factors were then investigated. A short FFQ for assessment of *n-3* LC-PUFA was designed and validated as no previous questionnaire was available. Red cell membrane fatty acids provided biomarkers of fatty acid status. These were used to validate the new FFQ and to investigate relationships of *n-3* fatty acids with atherosclerosis development.

Three dietary patterns were identified using principal component analysis. A healthy dietary pattern was associated with lower carotid artery intima media thickness in women in the highest compared with the lowest quintile for this dietary pattern score (mean difference: -0.07mm, 95% CI: 07 -0.1, -0.03, *P* = 0.002). A healthy dietary pattern was also associated with lower blood pressure,

heart rate, VLDL-cholesterol and triglyceride concentrations. Dietary patterns were not directly related to FMD.

Higher DHA status was associated with lower FMD in women (mean difference in absolute amounts: -0.08 mm, 95% CI: -0.1, 0.03; *P* = 0.001). Of other outcomes higher DHA status was associated with waist:hip ratio and fasting blood glucose only. Higher EPA status was associated with lower heart rate (mean difference -3 beats per minute. 95% CI: -7, 1; *P* = 0.01), and lower triglyceride and VLDL concentration.

In the RCT FMD was lower in DHA supplemented compared to control groups (mean difference in absolute amounts: -0.03 mm; 95% CI: -0.005 to -0.06 mm; *P* = 0.02). In secondary analysis this effect was confined to men. Of other outcomes, only triglyceride (mean difference: -28%, 95% CI: -40% to -15%; *P*<0.0001) and VLDL concentration were significant lower in DHA supplemented individuals compared to controls.

Collectively these findings suggest that protective effects of diet against the early development of atherosclerosis operate via reductions in conventional CVD risk factors, rather than via direct effects on endothelial function.

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Abbreviations in Text

AA	Arachidonic Acid
Ach	Acetyl Choline
AHA	American Heart Association
ALA	Alpha Linolenic Acid
Apo	Apolipoprotein
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CCA	Common Carotid Artery
CCA-IMT	Common Carotid Arterial Intima Media Thickness
CETP	Cholesterol Esther Transferase Protein
CHD	Coronary Heart Disease
CM	Chylomicrons
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DHA	Docosahexaenoic acid
DBP	Diastolic Blood Pressure
DRV	Dietary Reference Values
EDV	Endothelial-Dependent Vasodilatation
EE	Ethyl Esther
EF	Endothelial Function
eNOS	Endothelial Nitric Oxide Synthase
EPA	Eicosapentaenoic Acid
EPIC	European Investigation into Cancer
FBF	Forearm Blood Flow
FFA	Free Fatty Acid
FFQ	Food Frequency Questionnaire
FMD	Flow Mediated Dilatation
FO	Fish Oil
HbA1c	Glycosolated Haemoglobinn
HC	High Cholesterol
HDL	High density lipoprotein cholesterol
Hg	Mercury
HTG	Hyper Triglyceridaemia
I-CAM 1	Intracellular Adhesion Molecule
IMT	Intima Media Thickness
LA	Linoleic Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acid
LDL	Low Density Lipoprotein Cholesterol
Lp	Lipoprotein
LPA	Lipoprotein-a
LPL	Lipoprotein Lipase
LRNI	Lower reference nutrient intake
LT	Leukotrienes
MAFF	Ministry of Agriculture Fishery and Foods
MAP	Mean Arterial Pressure
MI	Myocardial infarction
MRI	Magnetic Resonance Imaging
MUFA	Monounsaturated Fatty Acid

***n3* HYP**	*n3 H*ealthy Young People
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
PCA	Principal Component Analysis
PDAY	Pathobiological Determinants of Atherosclerosis in Youth
PPL	Post Prandial Lipaemia
PUFA	Polyunsaturated Fatty Acid
PGE	Prostaglandins
PGI	Prostacyclins
PWV	Pulse Wave Velocity
RBC	Red Blood Cell/erythrocyte
RCT	Randomised Controlled Trial
RNI	Reference Nutrient Intake
ROS	Reactive Oxygen Species
RR	Relative Risk
SACN	Scientific Advisory Committee on Nutrition
SBP	Systolic Blood Pressure
sCAM	Soluble Cellular Adhesion Molecule
SFA	Saturated Fatty Acid
SOP	Standard Operating Procedures
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TFA	Trans Fatty Acid
TG	Triglyceride
TX	Thromboxanes
VAM	Vascular Adhesion Molecule
V-CAM-1	Vascular cell adhesion molecule 1
VLDL	Very Low Density Lipoprotein Cholesterol
VWV	Vessel Wall Volume
WHO	World Health Organisation

Diet and Endothelial Function in Healthy Young People

Introduction

Atherosclerotic cardiovascular disease (CVD) is the most common cause of death worldwide,[1] yet its pathogenesis and early development are only partially understood. Impairment of vascular endothelial function is central to the early atherosclerotic process. Endothelial dysfunction can be measured noninvasively and is evident in early life long before clinical signs of atherosclerosis emerge. Conventional CVD risk factors, present from as early as the first decade of life, strongly affect endothelial function (EF) and hence the development of atherosclerosis and CVD.

Risk factors for CVD most strongly implicated in the development of atherosclerosis include raised blood pressure (BP), dyslipidaemia, type 2 diabetes mellitus (T2DM) and obesity - all of which are strongly linked to diet. Diet is, therefore, an important modifiable risk factor for atherosclerosis. It is currently uncertain whether diet has direct effects on EF or whether dysfunction operates via CVD risk factors.

Certain dietary patterns and specific nutrients are proposed to be protective against the development of atherosclerosis. The strongest protective evidence is for 'healthy' or 'prudent' dietary patterns, characterised by higher intakes of fruits, vegetables and fish. Within healthy dietary patterns the strongest evidence is for the role of *n-3* fatty acids, in particular long chain polyunsaturated fatty acids (LC-PUFA) found in high concentrations in fish and fish oils.

A large body of epidemiological evidence supports the role of *n-3* fatty acids in primary prevention of CVD. Despite this, a causal association has not been established. Although randomized intervention trials have shown beneficial effects of *n-3* LC-PUFA supplementation, these trials have focused on secondary prevention and few studies have investigated the influence of *n-3* LC-PUFA on the primary prevention of atherosclerosis. Preliminary evidence now suggests that *n-3* LC-PUFA may have a favourable effect on EF or coronary artery disease

(CAD) and this could be one mechanism for their benefits in both the primary and secondary prevention of CVD.

The role of *n-3* LC-PUFA in the development of atherosclerosis was investigated with a randomised controlled trial (RCT) of *n-3* LC-PUFA supplementation in healthy young people. The primary outcome was EF measured in the brachial artery using non-invasive vascular ultrasound.

Assessment of background diet is essential in understanding diet-disease relationships. However, diet is difficult to measure accurately and quantitative measures of intake cannot easily be achieved. An alternative approach is the study of dietary patterns and relationships with health outcomes. Food frequency questionnaires (FFQ) provide information about habitual dietary intake and can be applied in dietary pattern analysis.

The aim of this PhD was to investigate relationships between habitual diet and the development of atherosclerosis. A generalised FFQ targeting all food groups was used to assess the whole diet, and from this, data were used to derive dietary patterns. Relationships between dietary patterns, EF and CVD risk factors were then investigated. A short FFQ for assessment of *n-3* LC-PUFA was designed and validated as no previous questionnaire was available. Red cell membrane fatty acids provided biomarkers of fatty acid status. These were used to validate the new FFQ and to investigate relationships of *n-3* fatty acids with atherosclerosis development.

Chapter 1

Cardiovascular Disease and its Risk Factors

The cardiologist's diet: If it tastes good, spit it out.

Section 1: Background

1.1 Cardiovascular Disease

The International Classification of Diseases, Tenth Revision (ICD-10) ranks circulatory diseases as the largest single cause of death across England and Wales (33% of all deaths in 2009: ONS, 2011).[2] Although rates vary across population groups, this pattern is consistent worldwide.[1] The term 'cardiovascular diseases' (CVD) encapsulates coronary heart disease (CHD – heart attacks), cerebrovascular disease (stroke), hypertension (raised BP), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. Internationally, the potential for preventing atherogenesis - the underlying pathology of the cardiovascular system in many diseases - is well understood and acknowledged (e.g., Kavey et al., 2003),[3] but requires changes in tobacco use, levels of physical activity and diet [1] and has thus far been unsuccessful.

1.1.1 Prevalence of Cardiovascular Disease

In its 2011 global survey, the World Health Organization (WHO) reported an estimated 17 million annual deaths from CVD.[1] Within this estimate, CHD accounted for 7.2 million deaths and 5.7 million were due to stroke. By 2020, WHO predicts almost 25 million people will die each year from CVD. The most recent Health Survey for England that addressed CVD and its risk factors (2006) reported a high prevalence of CVD that was similar in men and women (13.6 and 13.0% respectively).

1.1.2 Global Burden of CVD

The prevalence of CVD mirrors stages of epidemiologic transition. In areas where infectious diseases and malnutrition still prevail, e.g. Sub-Saharan Africa, rural India and South America, these diseases account for most deaths and CVD

mortality and morbidity is low (5-10%). As infectious diseases and malnutrition decrease, CVD and its risk factors increase.[4] Economically developing countries are now following similar trends to industrialized nations where rising income is accompanied by an increase in degenerative or "man-made" diseases that reflect effects of changing lifestyle on health.[5]

Whilst people in low- and middle-income countries are now more exposed to CVD risk factors they have less access to preventative measures, such as education and health care, than more affluent countries. In contrast, regions at an advanced stage of epidemiologic transition, including Western Europe, North America and Australia are able to focus more on prevention and delaying the onset of CVD to later in life.[6] Therefore, more people in low- and middle-income countries who develop CVD will die from it and at younger ages than in more affluent settings.

1.2 Clinical Signs of Cardiovascular Disease

Most CVD deaths result from CHD which targets the heart and blood vessels.

1.2.1 Coronary Heart Disease

Coronary heart disease is the single most common CVD (WHO, 2011)[1] and includes myocardial infarction (MI) and angina (chest pain on exertion). CHD commonly results from atherosclerosis **(Section 1.3)**, which may restrict the flow of blood to the heart muscle (myocardium) and compromise cardiac function. Clinical symptoms include shortness of breath and angina. Unstable arterial plaques are particularly dangerous as they may become dislodged from the artery wall and permanently block blood flow leading to ischaemia (relative or absolute restriction of blood flow leading to permanent tissue damage).[7]

1.2.2 Cerebrovascular Disease

Cerebrovascular disease results from damage to blood vessels supplying the brain, restricting blood flow and causing ischaemia. Thromboembolism (the dislodging of a blood clot (thrombus) which then occludes arteries serving the brain) is the commonest cause of ischaemic brain disease or stroke.[8]

Atherosclerosis increases the likelihood of fatal thromboembolism by decreasing arterial diameter.[9]

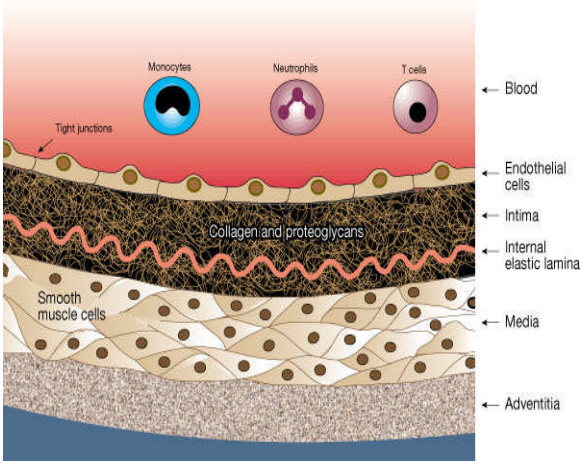
1.3 Atherosclerosis

Atherosclerosis is a disease in which arteries are narrowed as a result of lipid-rich lesions (plaques) that form in the blood vessel lining (endothelium), causing it to thicken. Understanding the atherosclerotic process requires knowledge of arterial wall structure.

1.3.1 Arterial Wall Structure

The structure of a normal artery consists of three distinct layers **(Figure 1-1)**. The innermost layer (intima) is very thin, comprising a matrix of extracellular tissue (proteoglycans and collagen). The intima is separated from the arterial lumen by a single layer of endothelial cells and connects to the medial layer (media) via a sheet of elastic fibres (internal elastic lamina). The media is made up mainly of smooth muscle cells. The outermost layer, the adventitia, consists of connective tissue, fibroblasts and smooth muscle cells.

Figure 1-1 Structure of a normal artery (nature.com)



1.3.2 Initiation of Atherosclerosis

The earliest stage in the development of atherosclerosis is a generalised disturbance of vascular function. This leads to the formation of 'fatty streaks' in the vascular endothelium of large arteries.[10] Over time endothelial dysfunction

results in thickening of the arterial intima and media and the eventual development of plaque.[11]

Fatty streaks arise when the vascular endothelium develops lesions that allow entry of 'foam cells' to the sub-endothelial space. Foam cells are formed from lipid rich cells that originate as monocytes, then differentiate into macrophages which take up lipid from the sub-endothelial space to become foam cells.[12] This accumulation of lipid-rich debris and the migration of smooth muscle cells to fatty streaks can lead to the development of more established fibrous lesions. These complex fibrous lesions can grow, forming large plaques that may block the arterial lumen, either partially or completely and impair or occlude blood flow.

The advancement of atherosclerosis is complex and involves interactions between foam cells, T-lymphocytes, smooth muscle cells, cytokines and numerous biological processes.

1.3.3 Role of Inflammation in Atherosclerosis

There is substantial evidence that chronic inflammation is central to the atherosclerotic process.[13] During chronic inflammation, modified lipoproteins, monocyte-derived macrophages and T-cells interact with normal cellular elements of the arterial wall and can trigger the atherosclerotic process. Raised concentrations of inflammatory markers are indicators of atherogenesis.

1.3.4 Role of Adhesion Molecules in Atherosclerosis

Entry of mononuclear cells to the endothelium is facilitated by specific adhesion molecules. These include: vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and selectins p and e.[14-18]

1.3.5 Role of Lipoproteins in Atherosclerosis

The main lipid contributors to atherosclerotic lesions are low density lipoprotein cholesterol (LDL) particles, in particular small dense LDL, remnant lipoproteins (or β-very low density lipoprotein (VLDL)) and lipoprotein a (Lpa). Lipoproteins

are taken up by macrophages and become foam cells. LDL receptors on the cell surface also have a major role in lipid accumulation.

1.3.6 Early Origins of Atherosclerosis

Research conducted into the origins of atherosclerosis shows conclusively that it begins in early life.[19-21] Development and progression of atherosclerosis in children and young people are influenced by risk factors such as obesity, hypertension, insulin resistance, T2DM, dyslipidaemia and smoking. For example the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) and Bogalusa heart studies both found strong relationships between CVD risk factors and early signs of atherosclerosis.[19, 22] Therefore, the primary prevention of atherosclerotic disease should begin in childhood, a strategy supported by public health policy, for example from the American Heart Association (AHA).[23]

1.4 Clinical Assessment of Atherosclerosis

Arterial stiffness and endothelial dysfunction are important risk factors in the aetiology of CHD. The vascular endothelium is the primary site of dysfunction in CVD and endothelial dysfunction is reported in patients with clinical CVD as well as in healthy individuals who may have CVD risk factors such as smoking, hypertension, dyslipidaemia, obesity and diabetes, but who have no clinical signs of disease.[24, 25]

Vascular function describes the regulation of blood flow, arterial pressure, capillary recruitment and filtration and central venous pressure. Mechanisms are complex and include interactions between intrinsic factors (e.g. nitric oxide [NO] and eicosanoids) and extrinsic systems (e.g. sympathetic and parasympathetic innervation, adrenaline, angiotensin, vasopressin). Components of vascular function include hypertension, arterial stiffness and endothelium-dependent vasodilatation. These are strongly associated with cardiovascular mortality and are therefore important risk factors that may be targeted with interventions including modifications to diet and lifestyle.[26-28] Endothelial function can be measured in peripheral arteries non-invasively using vascular ultrasound techniques and provides a surrogate measure of coronary artery function. The

most widely used technique is flow mediated dilatation (FMD), which is usually performed on the brachial artery **(Section 1.4.1.1)**. Studies have demonstrated a close relationship between endothelial function of the peripheral and coronary circulations.[29] Brachial artery FMD is also closely related to the angiographic extent of CAD.[30]

Arterial stiffness is the result of structural and functional changes in the blood vessel wall. Increased collagen and calcium deposition that occurs with vascular ageing reduces arterial elasticity.[31] The presence of fatty streaks or arterial plaques increases arterial intima media thickness (IMT) which also contributes to arterial stiffness. IMT thickness can be measured non-invasively with vascular ultrasound techniques **(Section 1.4.1.2)**. Together these structural and functional changes increase the risk of atherosclerosis**.**

1.4.1 Measurement of Vascular Structure and Function

Non-invasive vascular ultrasound techniques are established as valid and reliable tools that can be used clinically to identify individuals at risk of CVD.[32] Vascular structure is commonly assessed through measurement of common carotid artery intima media thickness (CCA-IMT).[33] Arterial stiffness can also be evaluated using measures of arterial compliance (distensibility)[34] and pulse wave velocity (PWV).[33] FMD is widely used to assess EF.[24, 35, 36]

1.4.1.1 Measurement of Vascular Function

Endothelial function can be assessed in either the coronary or peripheral circulation. For detailed methodology used in coronary artery endothelial function please see Lane, 2006.[37] Peripheral arteries provide a surrogate measure of coronary artery endothelial function and can be measured non-invasively using FMD. The brachial artery is most commonly used although carotid, superficial femoral and radial arteries have also been used. FMD is an endothelium-dependent process (EDV) where conduit arteries dilate in response to a flow stimulus. FMD is expressed as the change in diameter divided by the baseline diameter and can be measured with acceptable accuracy using high resolution ultrasound techniques and edge detection software.[38] During this process,

increased shear stress to the vascular endothelium, a single layer of cells lining the internal vascular wall, activates endothelial nitric oxide synthase (eNOS), an enzyme that prompts the intimal smooth muscle layer to relax and release vasodilators including NO. Ultrasound imaging can be used to measure changes in arterial diameter that occur during dilatory responses.[24, 35]

The artery is imaged using B (brightness) -mode ultrasonagraphy, a technique that produces a two-dimensional image from ultrasound echoes **(Figure 1-2).** Blood flow to the forearm is restricted using a high pressure cuff inflated to supra-systolic pressure **(Figure 1-3)**. Pressure is maintained for five minutes and occludes blood flow to the limb. Release of the cuff causes shear stress from ischemia-induced hyperaemia which stimulates the release of NO causing vasodilation. Ultrasound measurements, taken at baseline and during the peak vasodilatory response, provide a measure of endothelial function. Endothelial dysfunction is characterised by reduced vasodilation, increased permeability and activation of inflammatory pathways. For a detailed description of procedures for assessment of vascular structure and function in this PhD thesis please see **Chapter 5**.

Figure 1-2 Brachial Artery Image

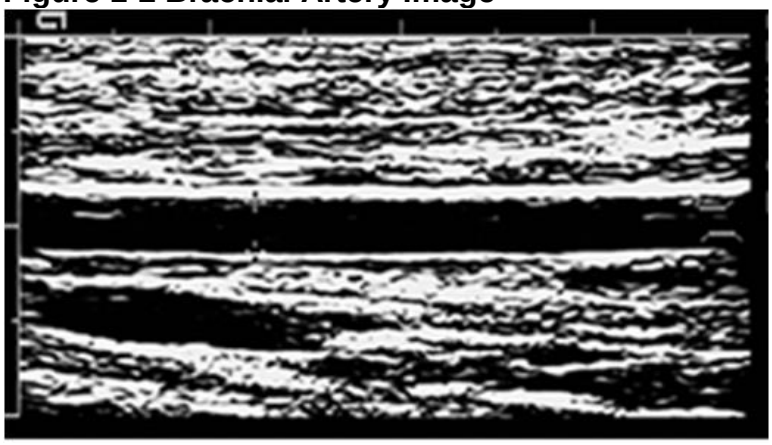
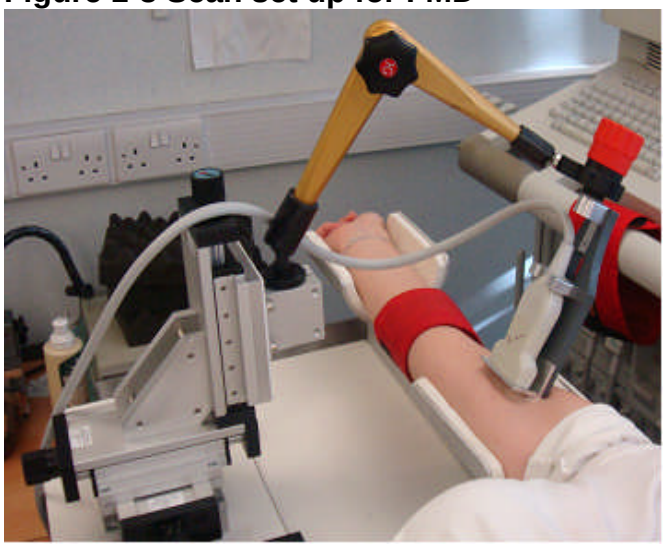


Figure 1-3 Scan set up for FMD



FMD is considered the most reliable technique for measurement of EDV.[36] Although there are several physiological, equipment-related and operator-related variables that can affect the reliability of this technique,[35] comparison with a "gold-standard" technique (intravascular ultrasound) has shown good reproducibility of baseline measurements.[39] Intra-individual variation within measurement session was acceptable (1.1% (extremes 0.06-2.0%) as was inter-operator variation (4.6%). However, intra-individual variation between sessions was considerable (13.9%).[39] Therefore the technique is recommended as suitable for group comparisons but may not be suitable as a follow-up parameter for clinical investigations in individuals.

Other older methods to measure EF include the L-arginine test,[40] measurement of forearm blood flow (FBF) using venous plethysmography[41] and digital pulse amplitude tonometry.[42]

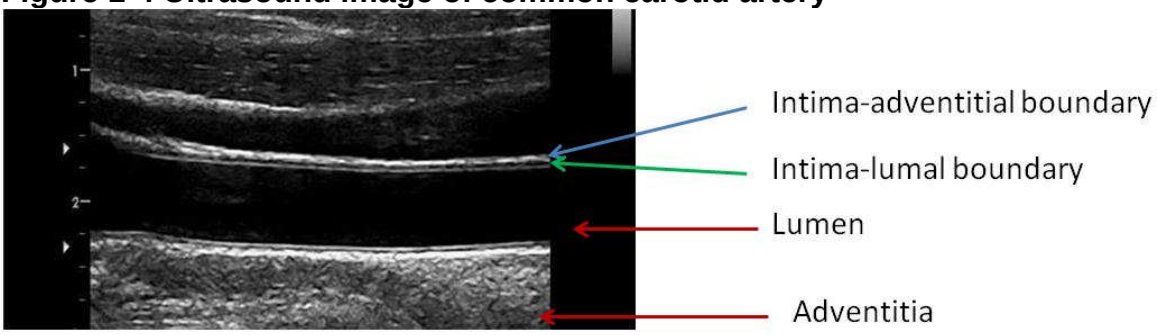
1.4.1.2 Measurement of Vascular Structure

Early atherosclerosis can also be detected through assessment of arterial stiffness or elasticity. Arterial stiffness is determined by the relationship between the change in arterial volume, the resultant distending pressure and the structural properties of the arterial wall e.g. collagen and elastin. Arterial stiffness can also be measured using non-invasive ultrasound techniques.[37] Arterial stiffness correlates closely with the extent of atherosclerotic disease[27] and with cardiovascular risk factors (even from early childhood).[19] Arterial stiffness is usually estimated from arterial distensibility, which is measured as the change in arterial diameter of a peripheral artery caused by pulse pressure.[43] A smaller change in arterial diameter in response to pulse pressure indicates a stiffer artery.

Alternatively, arterial stiffness can be assessed through measurement of the pulse wave velocity, i.e. the time it takes the pulse wave to travel a given distance (between pulse points). Typically, pulse wave propagation velocity between the carotid and femoral or carotid and radial pulses is measured.[44] Measurement is also carried out non-invasively using vascular ultrasound and magnetic resonance imaging (MRI).[45]

Sub-clinical atherosclerosis can be evaluated through the measurement of CCA-IMT using high resolution ultrasound. Increased carotid arterial wall thickness indicates the presence of fatty streaks or fibrous plaques. Population studies have found associations between increased CCA-IMT and progression of coronary artery disease.[46, 47] Therefore, measurement of CCA-IMT is prognostic of future cardiovascular events. A region of the common carotid artery, typically 1.0 cm proximal to the origin of the carotid bulb, is identified by B-mode ultrasonagrophy. The distance between the luminal-intimal surface and the media-adventitial interface represents the IMT.

Figure 1-4 Ultrasound image of common carotid artery



IMT is the distance between the intima lumen and adventia boundaries as depicted above.

Collectively, therefore, measures of vascular function, such as FMD and arterial distensibility, provide unique tools for the investigation, non-invasively, of the early origins of CVD. These measures are closely associated with cardiovascular risk factors and, at least in older individuals, show some prognostic value. Moreover, unlike other cardiovascular outcomes, these techniques can be used to measure the earliest stages of the atherosclerotic process in children, thereby avoiding some of the confounding effects of risk factors later in life on programming of adult CAD. Finally, early vascular dysfunction provides a potential mechanism that links factors in early life with later CVD.

1.5 Cardiovascular Disease Risk Factors

Risk factors for CVD include some that are immutable, such as age, gender and ethnicity and others that are modifiable. Key risk factors that are potentially modifiable include high BP, high blood cholesterol concentration, tobacco use, dietary factors, physical inactivity, T2DM and disposition. Targeting reduction of these risk factors offers an opportunity to minimise the risk of developing clinical CVD.

1.5.1 Non-modifiable Risk Factors

There are many CVD risk factors that cannot be changed. These are discussed below.

1.5.1.1 Gender and CVD Risk

In most developed countries, CHD is the leading cause of death in both women and men[1] despite CHD prevalence being higher in males than females.[1] Sex

differences in the pathophysiology, presentation and treatment of CHD are well recognized, but how these relate to CHD incidence is unclear.

For many decades, research investigating sex differences in CHD focused on a protective role of oestrogen, as CHD prevalence among pre-menopausal women was low.[48] The difference in CHD mortality between men and women narrowed considerably between 1994 and 2006 (ORs 1994 - 1995 1.44 versus 2004 - 2006 1.03), which may have been due to increased awareness of heart disease in women leading to improved diagnosis and management of risk factors.[49]

Environmental factors, such as diet and lifestyle may interact with gender to influence CVD risk. For example, secular changes in dietary fat consumption in England and Wales during the years 1921 to 1949 showed a similar pattern to changes in the sex ratio in mortality from CHD. Mean per capita fat consumption was positively correlated with the sex ratio for CHD mortality in men (r = 0.79, 95% CI: 0.70 - 0.86, P < 0.01) and inversely correlated to mortality in women (r = -0.30, 95% CI: -0.49 - -0.06, P < 0.01).[50]

1.5.1.2 Age and CVD risk

The age at which CHD first manifests varies according to gender and geographical location. Presentation with first MI has been reported as 9 years lower for men than women in all regions of the world.[51] Variations in age at first presentation of MI occur geographically with the youngest cases reported in South Asia (median age 53 years) and the Middle East.

1.5.1.3 Ethnicity and CVD risk

Differences in CVD risk and mortality are apparent between ethnic groups. For example, a survey investigating the 10 leading causes of death in the United States (US) reported the age adjusted death rates for CHD and cerebrovascular diseases were 1.3 and 1.5 times greater, respectively, for the black than the white population.[52] The reasons for this are unclear but risk factors are likely to be important contributors. The risk of having at least one uncontrolled CVD risk factor in non-white US veterans was twice that of white US veterans (95% CI: 1.8

- 2.3).[53] Differences in socioeconomic status may also be important.[54] Lower educational levels are consistently reported in association with CVD for non-white compared with white populations.[55] Evidence for disparities in CVD risk factors between white and non-white populations (British and Caribbean blacks, African–Americans and South Asians) continues to emerge.[56-58]

Men and women in Britain, whose families originate from India, have about 40% greater CHD mortality than nationals of European descent. This higher prevalence of CVD among migrant South Asians may be partly explained by socioeconomic differences.[59] However, when South Asian migrant populations are compared with indigenous controls, increases in obesity, cholesterol and insulin levels are notable, suggesting that nutritional factors influence increased CHD risk.[60]

In conclusion, non-modifiable risk factors do not act independently to increase CHD risk but are markers of modifiable risk factors.

1.5.2 Modifiable CVD Risk Factors

The worldwide prevalence of modifiable risk factors for CVD is extremely high - it is estimated that only 2-7% of the world's population have none and more than 70% have multiple risk factors.[4] Importantly, CVD risk is potentially modifiable; 8 preventable risk factors (tobacco use, physical inactivity, raised BP, raised blood glucose, raised blood cholesterol, alcohol use, high body mass index [BMI] and low fruit and vegetable intake) account for 61% of total CVD deaths.[51]

Until recently, knowledge of modifiable risk factors was based on studies conducted in populations of European origin. The INTERHEART study[51] investigated the strength of associations of CVD risk factors with health outcomes in a large multinational study designed to estimate risk in a more universally representative population, thus reducing bias introduced by regional and ethnic variations. Smoking (odds ratio [OR] 2.87 for current versus never) and increased levels of certain plasma lipoproteins (ApoB/ApoA1 [OR 3.25 for top versus lowest quintile]) were the two strongest risk factors, followed by

psychosocial factors (OR 2.67) history of T2DM (OR 2.37) and hypertension (OR 1.91).

1.5.2.1 Income Inequalities

Relationships between income inequality and CVD risk have been reported both between and within nations. Cross-national studies comparing nations undergoing recent economic/political transition report positive associations between higher income inequality and selected CVD risk factors, particularly BMI and obesity.[61] In the UK in 2006, for example, CVD and many of its risk factors were highest in low income groups.[62] This was particularly marked in younger people (< 35 years), where income was the greatest predictor of CVD. Greater disparity was seen for men: those in the lowest two quintiles for income had greater CVD risk compared with those in the highest 3 (prevalence > 20% versus < 18%) whereas women in the highest (prevalence 20%) and two lowest quintiles fared worse than those in the middle quintiles (prevalence 15%).

1.5.2.2 Obesity

Obesity is the consequence of a positive energy balance occurring when energy intake is higher than expenditure over a long time. Excess nutrition impairs systemic metabolic homeostasis and elicits stress.[63] It also activates an inflammatory process leading to increased circulating levels of pro-inflammatory cytokines, adipokines (hormone-like molecules produced by adipocytes) and other inflammatory markers. Adipokines (e.g. leptin, adiponectin, resistin and vistatin), and other chemical mediators such as tumor necrosis factor-alpha, interleukin-6, plasminogen activator inhibitor-1, lipoprotein lipase, acylation stimulating protein, cholesterol ester transport protein, estrogens, angiotensinogen and insulin-like growth factor-1 are present in increased concentrations in obese patients. These have various adverse effects on the cardiovascular system by creating a pro-inflammatory and prothrombotic state as well as causing endothelial damage and vascular hypertrophy.[64, 65] This results in a chronic active inflammatory condition that is associated with the development of a range of diseases. Obesity is known to directly impact CVD risk as well as indirectly through individual risk factors, as discussed below.

1.5.2.3 Direct Effects of Obesity on CVD risk

Obesity is an independent predictor of CAD, as observed in several studies.[66-68] For instance, the Framingham Heart Study, which followed the health of a 1948 born cohort and two subsequent generations in Framingham, Massachusetts, found that younger men (age < 50 years) who weighed more than 130kg demonstrated a two-fold increased risk of coronary disease compared with lighter men (< 110kg). For women, the risk increased 2.4-fold and remained after adjustment for other major CVD risk factors.[67]

The UK Health Survey for England (HSE) study (2006) found that abdominal obesity appeared to double the risk (OR 2.24) for acute MI.[62]

In the PDAY study, autopsy atherosclerotic plaques were highly correlated with the amount of abdominal fat and with BMI.[22] Obesity accelerates atherosclerosis decades before clinical manifestations appear even after adjustment for other risk factors including raised cholesterol, hypertension, smoking and increased glycosolated haemoglobin (HbA1c).[67] There are also direct effects of obesity on the heart and the cardiovascular system for example, cardiomyopathy prevalence is increased in obese individuals.[69] Several factors primarily caused by obesity, such as increased blood volume, elevated cardiac output, left ventricular hypertrophy and left ventricular diastolic dysfunction also play a role in causing heart failure.[70] Furthermore, the incidence of sudden cardiac death and arrhythmias is increased in obese individuals.[71]

1.5.2.4 Indirect Effects of Obesity on CVD Risk

Obesity is characterized by increased storage of fatty acids in an expanded mass of predominantly white adipose tissue. Central or abdominal obesity is marked by increased visceral adipose tissue (dispersed around the omentum, intestines and peri-renal areas,[72] which is mainly white adipose tissue. Visceral adipose tissue is more metabolically active than peripheral adipose tissue, and is involved in the control of metabolism through energy homeostasis, adipocyte differentiation and insulin sensitivity.[73] Furthermore, white adipose tissue regulates the production of anti-inflammatory molecules integral to metabolic and immune pathways. In

individuals with abdominal obesity, there is an increase in inflammatory markers as well as the occurrence of a prothrombotic state.[74]

Chronic inflammation, associated with obesity, results in overproduction of adipokines and hormones,[75] as well as insulin resistance, which in turn predisposes to the development of T2DM.[76] Both chronic inflammation and insulin resistance are strongly associated with endothelial dysfunction and the initiation and progression of atherosclerosis.[77]

In most obese subjects, plasma free fatty acid (FFA) levels are increased as a result of both overconsumption of dietary fat and increased lipolysis.[78] Increased FFAs inhibit insulin signalling and are thus implicated in the development of insulin resistance.[79] Furthermore, they may impair vasodilatory responses and are also implicated in endothelial dysfunction.[80] Obesity has been weakly associated with higher total cholesterol and LDL concentrations (r ~ 0.05 - 0.15) and more strongly with lower HDL, higher BP and insulin resistance.[81-83]

1.5.2.5 Blood Pressure

Hypertension is an important public-health challenge worldwide and has been identified as one of the most preventable causes of premature death.[84] Based on a single measurement greater than 140 mm Hg for systolic or 90 mm Hg for diastolic BP, approximately one in four adults, worldwide, would be classified as hypertensive.[85] Currently, this equates to about one billion individuals, and this number is expected to grow to > 1.5 billion, about one third of the world's population, by 2025. The greatest increases will be seen in low to middle income countries undergoing transition. Clearly this represents a large burden both to global health and economics.

There is a greater prevalence of hypertension in overweight compared with normal weight individuals. In men, hypertension prevalence is 15% in those with BMI <25 and 42% in those with BMI >30; in women, prevalence is 15% and 38%, respectively.[86] Therefore weight management has an important role not only in the prevention of metabolic syndrome but also in maintaining healthy cardiac function.

The effects of obesity on hypertension are proposed to operate via the renin-angiotensin-aldosterone and sympathetic nervous systems.[87] Increased activity of these systems may result in salt-sensitive hypertension.[88] The role of salt in hypertension is discussed in **Section 1.6.2.**

1.5.2.6 Insulin Resistance

Insulin resistance in adipocytes results in reduced uptake of circulating lipids and increased hydrolysis of stored triglycerides (TG). Increased mobilization of stored lipids in these cells elevates FFAs in plasma. Elevated FFAs reduce muscle glucose uptake, and increase liver glucose production, which both contribute to elevated blood glucose levels. Ultimately, if compensatory mechanisms are unable to maximize glucose utilization in this dearth of insulin, T2DM may result.

1.5.2.7 Dyslipidaemia

Dyslipidaemia is a syndrome characterised by overproduction or deficiency of specific lipoproteins and lipids. Presentation is usually marked by raised total serum cholesterol (TC) and changes in the ratio of potentially harmful cholesterol sub-fractions (non-high density lipoprotein cholesterol [HDL]) to potentially beneficial HDL concentration in the blood.

Triglyceride concentrations are often raised in tandem with other lipid fractions or may singularly indicate dyslipidaemia. Increased serum TG is often seen in association with high carbohydrate intake. In particular, high glycaemic index foods are implicated.[89] The combination of low HDL, increased TG-rich lipoprotein remnants and small dense LDL particles characterizes an atherogenic lipoprotein phenotype and increases overall CVD risk.[90, 91]

The presence of an atherogenic lipoprotein phenotype, particularly in combination with insulin resistance, contributes to increased risk of CHD. Small dense LDL particles have increased atherogenicity compared with larger LDL, possibly due to their greater propensity to be transported into the sub-endothelial space **(Section 1.3)**, their increased binding to arterial proteoglycans and high susceptibility to oxidative modification.[90]

1.5.2.8 Endothelial Dysfunction

Deterioration in EF and arterial stiffness are early events in the development of CVD **(Section 1.3)**. Endothelial dysfunction is reported in patients with all types of CVD, including CHD, peripheral arterial disease and chronic heart failure. Furthermore, patients who have CVD risk factors such as smoking, hypertension, dyslipidaemia, obesity and diabetes, but who have no clinical signs of disease, often display endothelial dysfunction.[24, 25] Lifestyle factors, including diet, have been shown to affect EF - in particular, diets high in fat are considered a risk factor for the development of atherosclerosis.[92, 93]

1.5.3 Cardiovascular Risk Factor Stratification

There are now 3 methods for assessing CVD risk in the UK: Framingham,[71] ASSIGN[94] and QRISK.[95] The Framingham risk assessment tool is limited by its development in an historic American population, and by the fact that it makes no allowance for family history of premature CHD or ethnicity. ASSIGN and QRISK are based on UK populations and do make allowance for social and lifestyle factors known to influence CVD risk. Current guidance for prevention of CVD in the UK is provided by the joint British Societies for Cardiology, Hypertension, Diabetes and Heart Health.[96]

1.5.4 Primary Prevention Strategies

Industrialization and technical advancements occurring over the last 50 years have led to changes in lifestyle that are strongly associated with increased CVD risk. Interventions aimed at reducing CVD risk through behavioural modification have demonstrated the strength of these associations. The Multiple Risk Factor Intervention Trial (MRFIT) was a multi-centre trial conducted by the US National Heart Lung and Blood Institute (NHLBI) that investigated the effect of a multifactor intervention programme on CHD mortality in 12,866 men with high CVD risk.[97-100] Men were randomly assigned either to an intervention providing treatment for hypertension, counselling and advice for cigarette smoking, diet and cholesterol reduction, or to their usual healthcare. Over a seven year follow-up, risk factor levels declined to a greater extent in the intervention group but no difference in CHD mortality was reported.[101] After longer term follow-up (10.5

years), however, CHD mortality was significantly lower (10.6% [*P* < 0.01]) for men in the intervention group.[102] This effect persisted at 16 years when 370 men in the intervention group and 417 men in the control group had died from CHD. This represents an 11.4% lower mortality rate for intervention versus control (95% CI: -23% - 1.9%).[103]

Caution should always be used when interpreting data from follow-ups that extend beyond the *a priori* stated study period, as validity may be compromised. For example, in many cases investigators do not continue to collect the same amount of data as in the trial itself because of cost considerations. Moreover, some patients will decline to participate in the extension or may change behaviours that are influential on the original trial outcomes. However, findings from intervention studies such as MRFIT and cohort studies including the Framingham Heart Study[67, 104] in the US and the Caerphilly Prospective Heart Disease Study in Wales[105] have directly influenced Government primary prevention strategies to manage the risks of hypertension and hypercholesterolemia.[106]

1.6 Nutritional Determinants of CVD Risk

The relationship between dietary factors and CVD has been a focus of research for many years. Evidence from the Seven Countries study, a 20-year study of about 12,000 men between the ages of 40 and 59 from 15 communities in Italy, Greece, Yugoslavia, the Netherlands, Finland, Japan and the United States, reported a clear and predictable pattern that linked diet to CHD prevalence.[107] Throughout the Mediterranean (Greece, and Southern Italy) and Asian (Japan) regions - where vegetables, grains, fruits, beans and fish predominate in the diet - heart disease was found to be rare. Conversely, populations consuming mainly red meat, cheese and other foods high in saturated fat - such as in the US and Finland - had high rates of CVD.[107] The Seven Countries Study was one of the first in humans to suggest a link between dietary fat and CHD risk. Death rates were positively related to high energy intake from saturated fatty acids (SFA) and negatively to dietary energy from monounsaturated fatty acids (MUFA).[108] Moreover, there were great differences in CHD mortality at similar blood

cholesterol levels, suggesting that fat intake, rather than cholesterol levels, may be linked to fatality.

The Seven Countries Study, together with data from animal models that showed diets high in cholesterol and saturated fat were associated with atherosclerosis, led Gordon (1988) to propose the diet-heart hypothesis.[109] Gordon postulated that dietary saturated fat and cholesterol played a primary role in the development of atherosclerosis and CHD in humans. This prompted more research in the form of prospective cohort studies and RCTs.

The relationship between diet and CVD is complex. Much confusion stems from the lack of definitive data regarding recommended diets and their potential health benefits. Overall, however, substantial evidence supports the hypothesis that dietary factors are important in the development of CVD and have been shown to have both protective and detrimental effects.[110] Relationships between dietary factors have been reported in association with a range of CVD risk factors including, obesity, hypertension, insulin resistance and dyslipidaemia. These are discussed in **Sections 1.6.1 – 1.6.4** below.

1.6.1. Dietary Fat and CVD Risk

Mechanisms for increased CVD risk are closely linked to and are often proposed to operate via obesity.[75] Fat is the most energy dense of the three macronutrients and is a major source of dietary energy. High intake of fat, particularly saturated fat, has been directly linked to obesity development in many studies. Fat is therefore highly implicated in CVD risk and has been the focus of many studies investigating relationships of diet and cardiovascular health.

1.6.1.1 Epidemiological studies investigating fat and CVD risk

The effects of dietary fat, both quantity and type, on the primary prevention of CVD have been investigated in numerous epidemiological studies.[111-127] In one of the earliest studies the Ireland-Boston Diet-Heart Study reported an association of dietary saturated fat with CHD mortality.[111] This large cohort study of about 1,000 middle-aged men used diet scores to estimate CHD mortality risk. Higher dietary scores for saturated fat and cholesterol were associated with increased CHD risk (relative risk [RR] 1.60). Conversely, high diet scores for fruit and vegetable intake were protective (RR 0.57). Although unable to demonstrate causality, this study provides support for the diet-heart hypothesis that first proposed a link between dietary fat and CHD; however, confounding cannot be ruled out because it is not possible to determine whether diet is a marker for other health behaviours.

A study of more than 10,000 health-conscious adults followed for 13 years in the UK reported an increased risk of CHD mortality with increasing intake of saturated fat and dietary cholesterol (death rate ratios in the third compared with the first tertile: 329 (95% CI: 150 - 721); 277 (95% CI: 157 - 796) respectively (*P* for trend <0.01).[115] Similarly, in the US, a lower intake of saturated fats was associated with lower CHD risk in elderly men.[125] The size of the beneficial effect was attenuated by fruit and vegetable consumption, suggesting that benefit was in part derived from other dietary components. It can be argued here that, as in the study of Mann (1997), high fruit and vegetable intake is a marker for health-conscious behaviour in general.[115]

Although many studies support an association between SFA intake and CVD risk confounding is a possibility and RCT data are needed to underpin public health messages.[111, 112, 115, 120, 125]

1.6.1.2 Randomised Controlled Trials - Fat and CVD Risk

Relatively few RCTs have investigated relationships between dietary fat and CVD.[128-132] Dayton and colleagues (1969) carried out one of the earliest intervention studies investigating effects of substituting saturated for unsaturated

fat.[129] The aim was to reduce serum cholesterol and minimize the risk of atherosclerosis. The intervention led to a 14% greater reduction in baseline cholesterol concentration compared with control. However, adjustment for baseline serum cholesterol concentration revealed that most of the effect was encountered in men with starting levels above the median (6.0 mmol/l). After 8 years follow-up, the incidence of all primary and secondary end-points was greater for the control compared with the intervention group (47.7% versus 31.3% [*P* = 0.02]*).*

The Women's Health Initiative Randomized Controlled Dietary Modification Trial delivered intensive behaviour modification sessions over a nine year period, aiming to reduce total fat intake to less than 20% of calories and to increase intake of fruit, vegetables and grains. At final follow-up (mean participation 8.1 years), modest effects were reported for some but not all CVD risk factors. Diastolic BP and LDL were reduced by 4.3% and 3.55mg/dl respectively. However, no differences in levels of HDL, TG, glucose or insulin were found.[131]

Clearly results from these studies are equivocal and it is questionable whether long-term interventions that impose severe restrictions on diets of free living individuals are justified.[131] It is possible that effects of interventions have a long latency phase as demonstrated in the MRFIT study where no difference was seen in CHD mortality after 6-8 years follow-up **(Section 1.5.4)**. However, at longer follow-up, of about 10 years, a significant reduction in CHD mortality was reported for the intervention group.[102]

1.6.1.3 Fat Quality and CVD Risk

Many observational studies have examined relationships between polyunsaturated fatty acids (PUFA), in particular LC-PUFA of the omega (*n*) *-6* and *n-3* series and CHD.[113-115, 117-119, 121, 123, 126, 127, 133] Observations by Bang (1976)[134] and Dyerberg (1975)[135] found associations between high fish consumption and reduced CHD risk among native Greenlanders, which focused studies on the role of *n-3* LC-PUFA. Protective effects of *n-3* LC-PUFA have been reported in some[114, 117, 119, 123, 126, 133, 136] but not all studies.[113,115, 118] The

role of *n-3* LC-PUFA in primary prevention of CVD, a key focus of this PhD, is discussed more fully in **Chapters 2 and 3**.

There is reasonably strong evidence to suggest *trans*-fatty acids (TFA) (found in some margarines, vegetable shortenings and processed baked and deep fried foods) are associated with increased CHD risk.[116, 137] In controlled metabolic studies, TFA have been shown to raise LDL and lower HDL plasma concentrations.[138-144]

1.6.2 Effects of Sodium Chloride on Blood Pressure

The incidence and severity of hypertension are affected by nutritional status and high intake of certain nutrients. For example, high sodium chloride (salt) intake is reported to predispose to hypertension,[145] and in many populations, salt intake exceeds dietary requirements. The UK Scientific Advisory Committee on Nutrition (SACN) issued a report in 2003 which concluded that there was evidence for a direct association between salt intake and high BP.[146] The report found that on average, people consumed 9g of salt per day and following their evidence review, SACN recommended that consumption of salt should be reduced by one-third to no more than 6g per day for adults.

Findings from large studies including the International Study of Macro- and Micro-nutrients (INTERMAP), a multi-national cross-sectional epidemiologic study of 4,680 men and women aged 40-59 from four countries (China, Japan, UK and US) and INTERSALT which studied over 10 thousand adults in 32 countries support SACN's findings but highlight important geographical variations. INTERMAP found that in China and Japan a higher incidence of stroke correlated with a higher intake of sodium whereas no such relationship was found among UK and US populations.[147]

Overall, however, intensive interventions, designed to reduce salt intake, have produced only minimal reductions in BP during long-term trials.[148] The role of dietary salt in hypertension development therefore remains unclear.

1.6.2.1 Nutrients and Blood Pressure

Some nutrients may be beneficial for BP. Potassium,[149] *n-3* PUFA,[150] vegetable protein[151] and vitamin D[145] have all been associated with BP lowering. Diets, such as DASH (Dietary Approaches to Stop Hypertension) that have been designed specifically to prevent or help in the treatment of hypertension demonstrate antihypertensive effects in the short term.[152] However, long-term effects of such diets are unknown and because many dietary changes are needed, compliance may be sub-optimal.[149] Furthermore, changing many aspects of diet makes it difficult to draw conclusions regarding the benefits of individual dietary components for BP.

The optimal macronutrient intake trial to prevent heart disease (OmniHeart) study compared three diets that differed in macronutrient composition: a carbohydrate-rich diet (58% carbohydrate, 15% protein and 27% fat), a higher protein diet (48% carbohydrate, 25% protein and 27% fat) and a higher unsaturated fat diet that had 10% more unsaturated fat and 10% less carbohydrate (48% carbohydrate, 15% protein, and 37% fat). All three diets reduced systolic BP compared with baseline. However, greater reductions were seen where carbohydrate was replaced with protein (1.4 mm Hg [P = 0.002]) or MUFA (1.3 mm Hg [P = 0.005]).[153] In summary, more controlled trials investigating effects of specific nutrients on BP are needed before definitive conclusions can be made.

1.6.3 Dietary Management of Type-2 Diabetes

Low-fat, high carbohydrate diets have been the cornerstone dietary intervention in the prevention and management of T2DM.[79] The biggest single risk factor for progression of insulin resistance to diabetes is obesity; low-fat, high carbohydrate diets are recommended to help achieve and/or maintain ideal bodyweight. Such diets are successful in the management of obesity and are consequently advocated as part of diet and lifestyle interventions that include advice for physical activity.[154]

More recently, alternative diets such as low-carbohydrate, high-protein diets[155] and low-glycaemic-index and low-glycaemic-load diets have been suggested as

equally effective in obesity management.[156, 157] However, the role of carbohydrate in the development of T2DM is controversial.

1.6.4 Dietary Management of Dyslipidaemia

Individuals with atherogenic dyslipidaemia are likely to be overweight. Therefore dietary interventions aim to both reduce obesity and improve the lipid profile. Until quite recently, dietary approaches for the management of dyslipidaemia have focussed on energy restricted diets with lower dietary fat, particularly saturated fat, being replaced with other macronutrients.

A lower intake of fat and, in particular, saturated fat is recommended for prevention of CVD.[158] In the UK, dietary requirements (dietary reference values – DRVs) for population groups are set by the Committee on Medical Aspects of Food and Nutrition Policy.[159] COMA recommends no more than 35% of total energy should come from fat with saturated fat contributing no more than 11% of total energy intake. However, these recommendations were published in 1991 and do not reflect recent evidence. The most recent dietary survey from the UK[160] reported that mean intake of total fat complied with the DRV in all age/sex groups except for women aged 65 years and over and men aged 65 years and over for whom, on average, total fat provided 35.9% and 37.1% food energy, respectively. Mean intakes of saturated fat exceeded the DRV in all age groups and in adults aged 19 to 64 years accounted for 12.8% food energy. Current dietary guidelines from the AHA recommend restricting consumption of fat to an upper limit of 30% of daily caloric intake.[161]

In the US, the National Cholesterol Education Programme (NCEP) advises a population approach targeting primary prevention and a clinical strategy for high risk individuals. For management of high cholesterol in adults a multifactorial lifestyle approach including reduced intake of saturated fats and cholesterol, inclusion of therapeutic dietary options for enhancing LDL lowering, weight reduction and increased physical activity is recommended.[162]

1.6.4.1 Low Fat Diets in the Management of Dyslipidaemia

There is a wealth of evidence to show that reductions in saturated fat offer the most effective dietary strategies for reducing total and LDL cholesterol levels, and hence cardiovascular risk.[163-165]

Reducing dietary fat to between 15 and 20% can reduce total and LDL serum cholesterol by 10 to 20%.[139, 165, 166] Very low fat diets that recommend less than 15% energy from fat do not achieve further reductions and can be difficult to adhere to. There are also concerns about adverse effects of low fat diets which if followed long-term may increase the risk of nutritional deficiencies, for example of essential fatty acids and fat soluble vitamins. Moreover, energy deficits arising from fat reductions are often replaced by increased carbohydrate which is also associated with adverse effects on the lipid profile. Reducing dietary fat can also achieve both short- and long-term weight loss but may not always benefit the lipid profile. For example, the Women's Health Initiative Dietary Modification Trial conducted among almost 50,000 overweight, post-menopausal diabetic females in the US compared effects of a fat-reduced diet and a typical US diet. Women taking part in an intervention to reduce total fat intake to 20% of calories and increase intake of vegetables/fruits to 5 servings/day and grains to at least 6 servings/day achieved a significantly greater weight loss, compared with controls. The difference was sustained 7.5 years after follow-up but no beneficial effects on the lipid profile were reported.[131]

1.6.4.2 Fatty Acid Composition and the Lipid Profile

The Lyon Diet Heart Trial provides support for the idea that specific fatty acids may be important mediators of the lipid profile. A diet enriched with *n-3* PUFA significantly reduced cholesterol compared with a Western-style diet.[167] Although this may have been partly an effect of the overall dietary pattern – a Mediterranean style diet (higher in fruits, vegetables, wholegrain cereals, nuts, seeds and legumes; and reduced in meat, eggs, dairy and non-oily fish) was advised for the intervention group – the Lyon investigators concluded that *n-3* PUFA was likely to be a major mediator of the protective effect provided by traditional Mediterranean diets.[168] However, the question remains open as

studies investigating relationships between dietary patterns and the early origins of CVD are lacking. Furthermore, there are no published reports of randomised trials investigating the effects of *n-3* fatty acids on the initiation of atherosclerosis in young healthy people, free from clinical CVD.

1.6.4.3 Carbohydrate in the Management of Dyslipidaemia

High carbohydrate diets are reported to have adverse effects on the lipid profile. In particular they produce a short-term increase in plasma TG concentration.[169, 170] This has led to the development of alternative diets that aim to reduce obesity and improve the lipid profile through carbohydrate restriction.

Examples of low-carbohydrate diets include the Atkins[171] and Zone[172] diets. Studies examining effects of low carbohydrate compared with low fat diets on CVD risk were most recently reviewed by Nordmann (2006).[173] A meta-analysis of RCT data found that low-carbohydrate diets led to greater weight reductions in the short term (6 months) (weighted mean difference, -3.3kg; (95% CI: -5.3 - -1.4kg). However, this was not maintained after 12 months (weighted mean difference, -1.0kg; 95% CI: -3.5 - 1.5kg). Triglycerides and HDL also changed more favourably in individuals assigned to low-carbohydrate diets (weighted mean difference -0.25mmol/L; 95% CI: -0.43 - -0.06mmol/L); and for HDL (weighted mean difference, 0.12mmol/L; 95% CI: 0.04 - 0.21mmol/L), but total and LDL cholesterol changed more favourably in individuals assigned to low-fat diets (weighted mean difference 0.14mmol/L; 95% CI: 0.03 -0.26mmol/L).

Low carbohydrate diets have been reported to have beneficial effects for the lipid profile including reductions in TG and LDL and increases in HDL.[174] However, concerns have been raised regarding effects of low carbohydrate on LDL sub-types. The most consistent finding in trials comparing low-carbohydrate with low-fat diets has been increased LDL concentrations.[175] Typically, small dense LDL is raised whilst TG levels are low to normal - a combination representative of an atherogenic lipoprotein profile.[90]

In general, diets that reduce carbohydrate but not total energy intake appear to be at least as effective as low-fat, energy-restricted diets in inducing weight loss

for up to one year.[173] However, it is not clear whether benefits for the lipid profile result from weight loss or through changes to the dietary macronutrient profile.[176]

1.6.4.4 Protein in the Management of Dyslipidaemia

High protein, energy reduced diets have been reported to increase satiety, induce weight loss and to have beneficial effects on the lipid profile. However, energy deficits are achieved through reductions in other macronutrients and in many investigations it has not been possible to separate effects of carbohydrate reduction and protein increase on appetite reduction and weight loss.

A series of weight-loss trials have manipulated protein and fat in energy-reduced diets, but kept carbohydrate constant in attempts to separate the individual effects of the diet components, but the results are not conclusive. For example, Luscombe-Marsh (2005) reported no significant differences in satiety, weight loss or plasma lipid concentrations when comparing high and low protein diets.[177] In contrast, increasing dietary protein from 15% to 30% of energy, at a constant carbohydrate intake, led to significant weight loss.[178]

1.7 Chapter Summary

Cardiovascular disease continues to be the major cause of death throughout the world. The epidemiologic transition occurring over the past few decades has lead to an increase in deaths from CVD in low- and middle- income countries whilst CVD prevalence represents a considerable health and economic burden in more affluent countries. CVD is potentially modifiable and a range of risk factors are implicated in its causation. Diet is an important risk factor and relationships between nutrition and CVD have been the focus of many epidemiological studies.

Only a small number of prospective controlled trials investigating relationships between dietary exposures and CVD risk and even fewer RCTs have been completed; therefore robust evidence is lacking. The strongest evidence for the role of diet in reducing CVD risk is an association with fruit and vegetable consumption and healthy dietary patterns.

Population studies, including The Seven Countries Study that linked dietary fat with increased CHD risk, were the first to propose a primary role of saturated fat and cholesterol in the development of atherosclerosis. Although dietary fat remains a plausible causal candidate in the aetiology of CVD, its specific role is undefined. This complicates conclusions regarding mechanisms through which dietary fat exerts effects on CVD risk. Both quantity and quality of fat are important. Saturated and *trans*-fatty acids are detrimental to CV health whereas unsaturated fats, in particular those of the *n-3* series confer protection.

In general, conclusions regarding specific effects of individual nutrients on CVD risk are difficult to draw. Nutrients are not eaten in isolation but are consumed together as part of diets. Similar nutrients coexist within foods and often cluster together. Individual diets are often characterized by high intake of foods with similar nutrient profiles. Individual dietary patterns often exclude food groups that have protective effects on health. For example, diets based on processed foods are typically high in fat, salt and sugar but low in fruit and vegetables. This high degree of co-linearity for both foods and nutrients within diets makes studies of relationships between single nutrients or foods and outcomes difficult to study. One approach, that is becoming increasingly popular, is to study dietary patterns in place of single foods or nutrients.

Atherosclerosis begins in early childhood and is the underlying pathological process in most CVDs. The earliest stage of atherosclerosis manifests as vascular dysfunction which can be measured non-invasively from as young as five years of age. Relationships of diet with vascular function have been the focus of recent research that is reviewed and discussed in the next chapter.

Chapter 2

Diet and Endothelial Structure and Function

Human beings do not eat nutrients, they eat food

2.1 Introduction

Evidence suggests dietary factors play an important role in modulating endothelial structure and function. Hence, relationships between diet and vascular structure and function (vascular health) have been the subject of both epidemiological and intervention studies.[93, 179] Improvements in endothelial health have been reported in association with many foods and beverages including fruit, vegetables, fish, tea and red wine and with nutrients such as *n-3* fatty acids, dietary vitamins C, D and E, carotenoids, flavonoids, alcohol and fibre.[180] A protective role of certain dietary patterns has also been reported in more recent studies.[92, 181]

2.2 Dietary patterns and vascular health

Until recently, dietary studies aimed to establish relationships between single foods or nutrients and EF.[182] However, the complexity of diet, which is a multifaceted exposure, is not well represented in such studies.[136] In the real world, free-living people eat foods and nutrients in a variety of combinations. The single food or nutrient approach therefore has limitations. Dietary pattern analyses, where foods and nutrients are summarized to produce patterns describing usual intake, may provide more useful information.

There are many examples of beneficial effects of more 'healthy' or 'prudent' dietary patterns compared with traditionally less healthy 'Western style' patterns on EF. For example, the Nurses' Health Study compared the effects of two major dietary patterns on EF in a large prospective study of more than 120,000 nurses registered in the US.[183] The study reported significantly lower levels of inflammatory markers of endothelial dysfunction for women who consumed a 'prudent' diet compared with those consuming a 'Western' diet. For example, the

Western pattern was positively related to e-selectin ($P < 0.001$) – a marker of endothelial dysfunction - whereas a prudent pattern showed an inverse association ($P < 0.001$).

2.3 Mediterranean style diets and vascular health

Mediterranean style diets, which are characterised by a high content of *n-3* fatty acids, flavonoids and antioxidants are associated with cardioprotective effects.[184] Interest in this area was prompted by the findings of the Seven Countries Study which showed that CHD mortality was two- to threefold lower in Southern Europe compared with Northern Europe and the US.[185] Support for a beneficial effect of Mediterranean style diets on CVD risk comes from meta-analyses that have reported significantly reduced CVD risk in association with close adherence to such diets (RR = 0.90; 95% CI: 0.87 - 0.93).[186, 187]

The importance of Mediterranean style diets for vascular health, as opposed to CVD, is less clear. This is largely because populations and reported outcomes differ between studies and diet affects other CVD risk factors. For example, in a study of healthy volunteers in Greece, improvements in FMD were reported only for subjects with central obesity following intervention to promote close adherence to their traditional Mediterranean style diet (2.05% increase in FMD; 95% CI: 0.97 - 3.13%) whereas no effect was found in the control group (-0.32%; 95% CI: -1.31 - 0.67%).[188] This suggests the effects of diet on FMD operate, at least in part, by decreasing classical CVD risk factors.

Other studies have investigated arterial structure rather than function as an indicator of CVD. For example, Shai and colleagues (2010) assessed changes in CCA-IMT and vessel wall volume (VWV) in participants of the randomized controlled DIRECT-Carotid trial **(Appendix 1-2, Table 2-2**).[189] This study compared two dietary strategies that aimed to achieve weight loss in middle aged men and women. Benefits for IMT and VWV were reported in association with both Mediterranean and low carbohydrate diets and operated largely through reductions in BP. This suggests that benefits for endothelial structure may also operate by reducing conventional CVD risk factors. The cardiovascular health benefits of specific nutrients within Mediterranean style diets have been less well

researched, largely because of difficulties in studying effects of single foods or nutrients within the whole diet.

2.4 Dietary components and vascular health

There have been attempts to identify benefits of single foods or nutrients using supplementation studies. However, there are few published reports of randomised trials of adequate sample size and duration to inform public health strategies in this area and no single nutrient or food has been identified to explain dietary effects on atherosclerosis and its risk factors. Epidemiological studies consistently report associations of *n-3* LC-PUFA with a lower risk of CVD.[190] This has lead to these fatty acids becoming the focus of much research into dietary determinants of CVD risk.

n-3 fatty acids have been shown to protect against secondary CVD, for example in reducing the risk of subsequent heart attack, in both epidemiological[135, 191-194] and experimental studies (**Chapter 3, section 3.5.1)**.[132, 195, 196] However, there are few data from large scale RCTs investigating the role of *n-3* PUFA in primary prevention of CVD. *n-3* fatty acids are the focus of **Chapter 3** where their role in relation to cardiovascular health is more fully discussed.

A comprehensive literature review was carried out of studies investigating the effects of diet on vascular structure and function. The aim of the review was to summarise evidence from healthy people, free from CVD.

2.5 Comprehensive Review of Diet and Vascular Health

2.5.1 Comprehensive Review – Methods

A systematic search of MEDLINE was carried out for published articles of studies investigating dietary exposures in relation to vascular structure and function in healthy individuals published between January, 1992 and September, 2012. This period was chosen to capture the earliest non-invasive investigations of EF which commenced in 1992.[24]

The search strategy was developed from concepts within the research question according to the PICOS system.[197] Search terms included MeSH and free text terms identified as keywords in articles investigating diet and vascular health. Key terms included "vascular structure," "EF," "FMD" "carotid intima-media thickness," "pulse wave velocity", "atherosclerosis," "coronary artery disease," "cardiovascular risk". Full details of the search strategy can be found in **Appendix 1-1**. A hand search of bibliographies of included studies was also carried out and relevant experts were consulted to identify any omitted studies.

Inclusion of studies was limited to papers written in English and published in a peer-reviewed journal between January, 1992 and June, 2012. Only studies which met specific inclusion criteria **(Box 1)** were assessed.

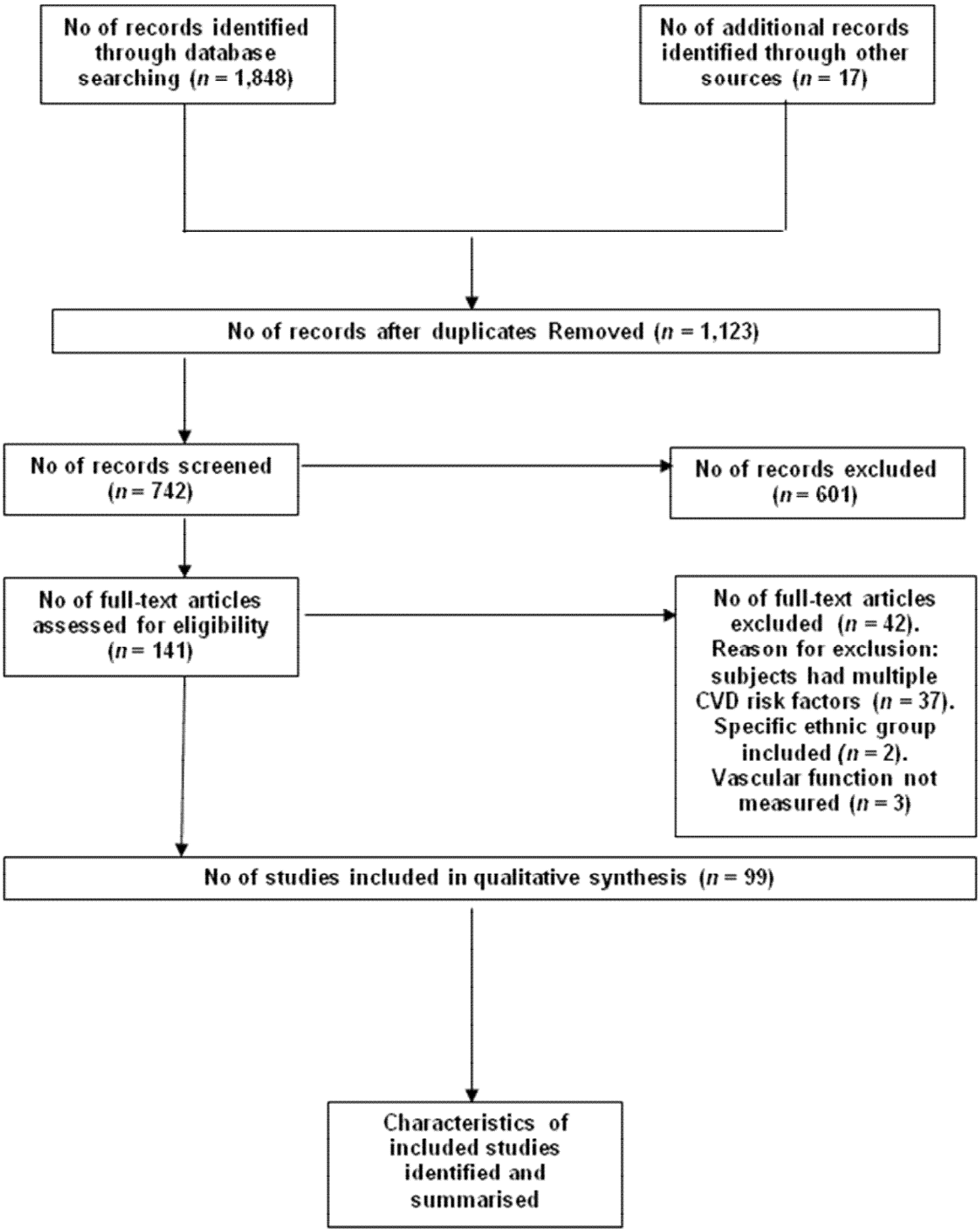
Box 1 Study inclusion criteria

- Written in English
- Published in peer-reviewed journal
- Includes details of dietary exposures
- Includes assessment of relationship of dietary exposures to measurements of vascular structure (e.g. IMT, arterial distensibility or pulse wave velocity) or endothelial function (e.g. EDV, EIDV or FMD).
- Volunteers free of clinical CVD and multiple risk factors
- Volunteers not taking regular medication

2.5.2 Results

Figure 2-1 presents the number of studies evaluated and excluded through the stages of the literature review. The MEDLINE search yielded 1,848 references. A further 17 articles were identified through other sources including online articles and reports from conference proceedings. Screening of abstracts and reference lists identified 742 potentially relevant articles of which 99 met the inclusion criteria. The main reasons for exclusion were pre-existing CVD or multiple risk factors.

Figure 2-1 Flowchart summary of literature search



Thirty-five reports were from studies investigating effects of short term dietary interventions following ingestion of single meals, dietary supplements, food items or beverages.[80, 198-231] Most of these (*n* = 23) considered the effects of fat and/or fatty acids[80, 198-204, 206, 208, 209, 212, 214-219, 221, 222, 226, 227, 230] two of which also considered the addition of red wine to diets of varying fat content.[201, 203] The

remaining 12 studies investigated caffeine,[210, 211, 213] flavonoids [223 224, 231] vitamin C,[225, 228] champagne,[229] cocoa,[207] red wine[205] and the NO precursor amino acid - L-arginine **(Appendix 1-2, Table 2-1)**.[220]

Thirty-six studies considered longer term interventions (**Appendix 1-2, Table 2-2**).[188, 189, 233-266] As with shorter term studies most of these reports (*n* = 21) were of interventions comparing diets with differing fat and/or fatty acid composition.[234, 235, 237, 238, 240, 242, 243, 245-248, 250, 252, 253, 255-257, 260, 261, 266, 267] Most of the remaining 15 studies investigated relationships between single dietary components and endothelial health. These included, lycopene,[244, 263] flavonoids,[239 249, 259, 265] vitamins D[258] and E,[251] pistachio[264] and walnuts.[236] Five studies investigated effects of dietary interventions. Four of these studied relationships between the Mediterranean diet and vascular health,[188, 233, 246, 254] and one considered the DASH diet.[241]

Twenty eight studies reported results of observational studies.[183, 268-294] All these studies provided data relating to nutrient or food intake or dietary patterns and a measure of vascular structure or function **(Appendix 1-2, Table 2-3)**.

Six studies considered relationships between dietary patterns and vascular health. Four related dietary patterns to measures of vascular structure[268, 278, 285, 295] and two considered markers of EF.[287. 183] No study investigating dietary patterns considered FMD as an outcome. One study assessed adherence to the Mediterranean diet in relation to PWV, a measure of arterial stiffness.[290]

Six studies considered fish and/or *n-3* LC-PUFA intake.[270, 274, 275, 282, 286, 293] Thirteen studies investigated micronutrients including Zinc,[272, 294] Magnesium,[272, 283] vitamins C,[272, 279, 284] D[269, 276] and E[272, 284] and two assessed flavonoids[280, 292]. Rissanen (2000 and 2003) investigated relationships between lycopene and vascular structure.[288, 289] The remaining 3 studies considered foods. Fruit and vegetable consumption was the subject of two of these studies[277, 291] and the third investigated dairy foods.[271]

2.5.2.1 Effects of fat on endothelial health

Studies investigating effects of fat intake on endothelial health were mainly short term and most investigated the acute effects of fat on EF. Overall, studies directly comparing high with low fat meals consistently reported detrimental effects of high fat meals on EF.[80, 198, 199, 203, 204, 209, 212, 215, 217, 219, 230] Studies that compared effects of meals with differing fatty acid compositions were less conclusive. For example, Berry (2008),[221] Vogel (2000)[204] and Ong (1999)[200] reported that FMD decreased more following a meal high in MUFA than with a high SFA meal. Four short-term studies considered effects of meals rich in PUFA on FMD. Three of these reported improvements in FMD[222, 226, 227] and one study (Nicholls, 2006) found no difference in FMD according to meal fat type.[218]

Longer term interventions to alter dietary fat consumption have mainly compared high fat diets with low fat Mediterranean style diets.[188, 233, 241, 246, 248, 252, 255] FMD improved with low fat Mediterranean style diets in three studies[188, 246, 248] but no benefits were seen in two studies.[241 252] Four interventions investigated relationships of Mediterranean style diets with vascular health either alone or with additional components. For instance, two studies considered the effects of interventions that aimed to improve compliance with Mediterranean style diets,[188, 189] one study compared a Mediterranean style diet with the usual diet in a Swedish population,[233] and another investigated additional benefits of pistachio nuts.[254] One study considered the effects of the Dietary Interventions to Stop Hypertension (DASH) intervention (a programme that aims to reduce hypertension through dietary intervention) on FMD.[241]

2.5.2.2 Effects of n-3 fatty acids on endothelial health

Relationships between *n-3* LC-PUFA and vascular health were reported in 15 longer term (two weeks to two years; **Appendix 1-2, Table 2-2**) controlled intervention studies among healthy human subjects.[234, 235, 237, 238, 240, 242, 243, 245, 247, 250, 253, 256, 260, 261, 266] Methods used to assess vascular function included vasodilation of peripheral arteries in the micro-[235, 243, 256] and macro vasculature.[204, 242, 253, 256, 266] Three studies used FMD to assess EDV in large blood vessels considered to reflect coronary artery function.[242, 253, 256] Six studies

investigated relationships between diet and markers of endothelial activation.[234, 237, 238, 247, 250, 261] Most studies were conducted in small samples (< 50 participants) and results are conflicting.

Six studies reported no benefits of fish oil supplementation for vascular health.[237, 242, 245, 250, 253, 255, 260] Four of these were RCTs where sample size ranged from 38–312.[242, 250, 253, 260] Only two of these studies assessed EF using FMD.[242, 253, 255] Eight studies reported benefits for vascular health: improvements were reported for FMD[256] and EDV in the microvasculature[235, 243, 245, 260, 266] or for concentrations of vascular adhesion molecules (VAMs).[247, 261] Two studies that measured VAMs reported benefits of *n-3* supplements for older men but adverse effects in younger men.[247, 261] This finding is supported by Cazzola (2007) who, in a study of 93 younger men (mean age 26 years) reported increased VAMs after supplementation with 4g EPA and DHA combined for 12 weeks.[234]

Several observational studies investigated associations of *n-3* PUFA with vascular health.[270, 273-275, 282, 286, 293] Djousse and colleagues (2003) found a lower prevalence of plaques and lower carotid IMT in a large cohort (*n* = 1,575) of healthy Caucasians in association with ALA, the parent *n-3* fatty acid. However, no benefits were found in association with fish or *n-3* LC-PUFA in this study. Most other studies found benefits to measures of vascular structure or function with fish and/or *n-3* fatty acid consumption. One report from the MESA study reported lower FMD for women with the highest fish intake.[270]

2.5.2.3 Effects of flavonoids on endothelial health

Eight intervention studies that investigated relationships of dietary flavonoids with endothelial health assessed either brachial artery FMD [207, 223, 224, 231, 232, 239, 259, 265] or microvascular reactivity.[249] Three studies considered tea consumption,[223, 224, 239] all of which were of small sample size (≤ 30) and were short duration studies.[223, 224, 239] All studies reported improvements in FMD following tea consumption ranging from 4–7% increases in FMD from baseline. Effects were seen at doses equivalent to usual dietary intake (i.e. with 100g flavonoids as found in one standard mug of tea).

The remaining 5 studies used various flavonoid rich foods including apples and spinach,[231] grapes,[265] oranges (high in hesperidin)[249] cocoa[207] or soy.[259] All studies reported improvements in FMD, however, most were of short duration and small sample size. The one large scale RCT that investigated effects of flavonoids reported a reduction in FMD for men following 3 months supplementation with soy protein, providing 118 mg isoflavones per day.[259]

Support for a protective effect of flavonoids on vascular health comes from two large observational studies. Landberg (2010) reported from the US nurses study that women in the highest quintile for flavonoid intake had lower concentrations of VAMs compared with those in the lowest (*P* for trend = 0.012).[280] Similarly, a study of 433 post-menopausal women from the Netherlands reported lower PWV in women in the highest compared with the lowest quintile of flavonoid intake (*P* for trend = 0.07).[292]

Four studies considered the acute effects of wine on vascular health,[201, 203, 205, 229] and of these, three found protective effects on vascular function. In one study, the deleterious effects of a high fat meal were reduced when the meal was given with red wine.[203] Champagne wine led to increased endothelium-independent but not endothelium-dependent vasodilatation 4–8 hours following consumption.[229] No such effect was seen when a beverage matched for alcohol, carbohydrate and fruit content was given in one study.[201, 229] However, improvements for FMD were seen following consumption of red wine without alcohol in another study.[205]

2.5.2.4 Effects of caffeine on endothelial health

Three studies considered the effect of caffeine on vascular health.[210, 211, 213] All three reported moderate increases in measures of aortic stiffness at doses ranging from 80-300mg/d.

2.5.2.5 Effects of lycopene on endothelial health

Two studies reported results of interventions with lycopene, a carotenoid found in high concentrations in tomatoes and other red/orange fruits and vegetables, to

improve vascular health.[244, 263] Both studies measured inflammatory markers in middle-aged subjects following interventions to increase lycopene intake for similar time periods. Thies (2012) reported no difference in markers of vascular function or measures of arterial stiffness in a UK population following a 12-week intervention period.[263] However, Kim (2011) reported reductions in inflammatory markers that correlated with increased lycopene concentrations.[244] Conclusions are difficult as the studies varied with respect to population and outcomes studied. However, further support for a protective role for lycopene in vascular health comes from two large scale observational studies in Finland. Middle aged men were found to have higher CCA-IMT in association with low plasma lycopene.[288, 289]

2.5.2.6 Effects of micronutrients on endothelial health

This review identified 11 studies investigating relationships between micronutrients and vascular health. Four of these were intervention studies[225, 228, 251, 258] and the remainder were observational studies.[269, 272, 276, 279, 283, 284, 294] Nutrients investigated included vitamins C, D and E and minerals zinc and magnesium.

Three small studies considered the relationship between vitamin C (ascorbic acid) and measures of vascular function.[225, 228, 284] One study reported improved blood flow following ascorbic acid infusion during exercise.[228] The second intervention study found no benefits for FMD following a large oral dose of ascorbic acid.[225] One small observational study that compared FMD in healthy men reported a 20% lower FMD for men with lower vitamin C intake. However, effects were confined to obese men. One large epidemiological study does, however, provide evidence to support the hypothesis that vitamin C is protective for vascular health: a report from the Atherosclerosis Risk in Communities (ARIC) study found a significant inverse association between vitamin C intake and CCA-IMT in more than 10,000 middle-aged men and women.[279]

Studies of vitamin D suggest that supplementation provides benefits for individuals who have low levels. An intervention to increase vitamin D status in 23 asymptomatic vitamin D deficient subjects reported improvements in FMD

following supplementation with 300,000 IU (7.5 µg) of vitamin D given intramuscularly once monthly for 3 months.[258] The role of vitamin D in vascular health is supported by observational study findings that higher FMD is associated with higher status.[269, 276]

Only one trial investigating the role of vitamin E in vascular health was identified. Plasma vitamin E was not related to PWV, a measure of vascular structure, in 20 post menopausal women supplemented with 400 IU (268 mg) alpha tocopherol for 10 weeks.[251] This conflicts with the epidemiological Atherosclerosis in Communities (ARIC) study which found a significant inverse relationship between vitamin E and CCA-IMT in women.[279]

There were no interventions of mineral supplementation to improve vascular health. However, habitual zinc intake was inversely related to CCA-IMT in a large study of more than 4,000 healthy adults in South Korea. Men and women in the highest quintile for zinc intake had a reduced risk of subclinical atherosclerosis (5^{th} versus 1^{st} quintile, OR 0.64, 95% CI 0.45 - 0.90).[296] Similarly, dietary magnesium intake was inversely associated with CCA-IMT in women participating in the ARIC study.[283]

2.5.2.7 Effects of diet on endothelial health

There were four reports of whole diet interventions to improve vascular health.[188, 233, 241, 246] Three studies assessed effects of Mediterranean style diets[188, 233, 246] and one considered the DASH diet.[241] Two studies investigated effects of the Mediterranean diet on EF with conflicting results. In a randomised cross-over study, Ambring (2004) found no benefits for FMD in a group of healthy individuals who followed a Mediterranean style diet for four weeks compared with controls consuming their usual Swedish diet.[233] In contrast, Rallidis and colleagues (2009) reported an increase in FMD for an intervention group who were instructed to follow a Mediterranean style diet under close supervision for two months.[188] In a third study, markers of endothelial activation were reduced in a group of 20 healthy subjects randomised to a Mediterranean style diet for 4 weeks. Endothelial progenitor cells, indicative of a healthy endothelium, were higher

following the intervention.[246] Hodson (2010) reported no change in FMD for a group of 27 healthy adults following the DASH diet.[241]

Two studies considered in this review investigated effects of nuts: pistachio[254] or walnuts[236] on endothelial health. The addition of pistachio nuts to a Mediterranean diet was reported to improve FMD in 32 healthy young men. Compared with Mediterranean diet only, a pistachio enriched diet produced a 30% increase in FMD (P = 0.002). Walnuts had no effect on arterial stiffness, in a group of 30 healthy males randomised to receive a 15g/day supplementation of walnuts for 4 weeks.[236]

The two remaining studies considered the effects of garlic, an alanine rich plant (Turner, 2004)[264] and L-arginine, the precursor to NO.[220] Garlic supplements, providing 10 mg/day aliin (equal to about 3 garlic cloves) did not improve arterial stiffness in 75 healthy volunteers. Mariotti (2007) manipulated amino acid content of meals to investigate the role of dietary L-arginine in NO production. Dietary L-arginine had no effect on endothelial structure or function.[220]

2.6 Discussion

The current evidence from this review of the relationship between diet and vascular health suggests that healthy dietary patterns, mostly characterised by higher intakes of fruits, vegetables and fish protect against atherosclerosis development in healthy people **(Table 2-3)**.[183, 268, 278, 285, 287, 295] Large scale epidemiological studies have shown reductions in arterial stiffness,[278, 285, 295] markers of endothelial dysfunction including PWV[268] and inflammatory markers.[183, 287] It is not possible to conduct intervention studies of dietary patterns, which are described post priori, however, studies of specific diets support a protective role of healthy dietary patterns.

2.6.1 Diets and Dietary Patterns

Mediterranean style diets are shown to confer protection against the development of atherosclerosis in observational[290, 291] and experimental studies.[188, 248, 297, 298] Improvements in EF following a Mediterranean style diet for a brief period have

been reported in subjects at low[188, 248] or moderate[297] risk of CVD and in patients with T2DM.[299] Studies in individuals free from CVD risk factors have not always reported benefits for EF.[233] However, differences in study design including intervention duration, dietary assessment methods, outcomes, sample sizes and population health status make conclusions difficult to draw.

As with dietary patterns, exact mechanisms through which specific diets protect against CVD are difficult to elucidate. This is largely because diets are complex, contain many components and affect other CVD risk factors. Mediterranean style diets may benefit vascular function indirectly through reductions in classical CVD risk factors. For example, the DIRECT-carotid study reported regression in carotid IMT in middle-aged men following either a Mediterranean style diet or one reduced in fat or carbohydrate. However, adjustment for confounders suggests that this was mediated mainly by a weight loss-induced decrease in BP.[189] Other studies have reported benefits of a Mediterranean diet for the lipid profile, which may in turn affect endothelial structure and function.[297, 300] The mechanisms through which specific diets improve vascular health are therefore unknown.

2.6.2 Dietary Components

Experimental studies that have intervened with specific dietary components provide evidence for some foods and nutrients that characterise healthy diets and dietary patterns. This review found many dietary and nutrient interventions that modified vascular structure and function in healthy people to varying extents **(Appendix 1-2, Tables 2-1 & 2-2)**. Acute ingestion of high fat meals directly impaired vascular function in most studies. The mechanism for FFA related endothelial dysfunction cannot be drawn from this review. However, it is likely that the elevated and extended post prandial lipaemia that is often reported following high fat meals plays a role **(Chapter 3, Section 3.7.4.5)**.

2.6.2.1 Dietary Fat

Diets high in fat, and particularly those high in saturated and/or *trans* fats, are considered a risk factor for the development of atherosclerosis.[92, 93] High saturated fat intake leads to increased circulating lipoproteins and free fatty acids

which may stimulate pro-inflammatory pathways and increase oxidative stress **(Chapter 3, Section 3.7.4)**. Furthermore, studies have shown that high fat meals induce endothelial activation, increase inflammatory cytokine production and may also have independent adverse effects on the vascular endothelium.[301] Studies investigating the effects of fat intake on EF have mainly been short term as the adverse effects on health from habitual high fat diets are well recognised.[302] Long term RCTs that increase dietary fat intake would be unethical and thus, evidence underpinning the role of long term high fat consumption on vascular health only comes from observational studies.

Endothelial activation increased in several studies in response to high fat consumption indicated by increased serum concentrations of inflammatory cytokines and VAMs. Another possible explanation for adverse effects of high fat meals on EF is increased production of reactive oxygen species (ROS) by FFAs. Ceriello (2002) reported FFA directly enhanced ROS production after consumption of a high fat meal and was closely associated with endothelial dysfunction.[303] The proposed mechanism for the effect of FFA on ROS production is via up-regulation of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH). Inhibition of NADPH by antioxidants has been shown to prevent FFA-induced endothelial dysfunction.[304]

2.6.2.2 Fatty acid composition

The type of fat is important in the effect of diet on vascular health. This review found that specific fatty acids have different effects on EF. In particular, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to improve EF at least *in vitro*[305] in short-term post prandial studies of acute ingestion[222, 226, 227] and in longer-term studies among healthy individuals[235, 243, 245, 247, 256, 260, 262, 266] The role of *n-3* fatty acids in vascular protection is as yet unclear. However, the physiological effects of EPA and DHA that reduce vascular reactivity and maintain vascular integrity through the production of less inflammatory mediators are likely to be important **(Chapter 3, Section 3.4.3.1)**.[306]

Both EPA and DHA reduce cardiac arrhythmia.[307, 308] EPA may also protect against thrombosis[309, 310] through the inhibition of platelet cycloxygenase.

Lowering plasma triglycerides,[311, 312] increasing endothelial nitric oxide[93] and attenuating inflammation[313] have also been proposed as additional mechanisms by which *n-3* fatty acids might favourably influence the risk of atherosclerosis.

Consumption of nuts, rich in the *n-3* parent fatty acid alpha-linolenic acid (ALA) **(Chapter 3, Section 3.4.3)** has been reported to benefit EF in healthy subjects. Adding walnuts[236] or pistachio nuts[254] to diets was beneficial for FMD in two short term small trials. Results from such small non-randomised studies cannot inform dietary strategies but rather generate hypotheses for future investigations.

Observational studies support the findings of intervention trials and report associations between higher consumption of fish or *n-3* LC-PUFA with reduced risk of atherosclerosis.[275] For example, the Multi-Ethnic Study of Atherosclerosis (MESA) found significant associations between fish intake and markers of endothelial inflammation and activation. *n-3* LC-PUFA intakes were inversely associated with plasma concentrations of biomarkers independent of other CVD risk factors.[275]

Several studies investigating associations of *n-3* LC-PUFA with CCA-IMT report conflicting findings. For example, Djousse and colleagues (2003) found no associations of fish or *n-3* LC-PUFA consumption with IMT in a large cohort (*n* = 1,575) of healthy Caucasians.[273] Conversely, Ebbesson and colleagues (2008) reported a protective effect of fish intake on IMT in Alaskan Inuit, age 35 and older (*n* = 686).[274] These two studies differed in many ways. Djousse (2003) studied individuals participating in a North American study (NHLBI Family Heart Study) who were chosen either at random or on the basis of a higher than expected risk of CAD. Ebbesson (2008) studied an indigenous population of Inupiat who were largely village dwellers with a hunter-gatherer existence. Therefore, comparisons between these two studies are difficult to make. Reliable conclusions cannot be drawn from only two studies and further research is needed to further to define the relationship between *n-3* LC-PUFA consumption and vascular health.

2.6.2.3 Polyphenols

Previous studies suggest that EF could be protected by the addition of nutrients with antioxidant functions, such as polyphenols, to high fat meals. For example, red wine was shown to attenuate the adverse effects of a high fat diet on FMD.[203] Red wine contains several polyphenolic compounds that have antioxidant properties.[314] Consumption of polyphenols from various sources is associated with a decreased risk of CVD.[315] Furthermore, vitamins with antioxidant functions have also been reported to protect against endothelial dysfunction. For example, Plotnik (1997) reported that impaired EF observed after a high-fat meal could be prevented by concomitant treatment with vitamins E and C.[316] This suggests that postprandial endothelial dysfunction could be mediated by an oxidant mechanism and that consumption of polyphenols and other antioxidants could also have a beneficial effect. Support for this comes from mechanistic studies in animal models. For example, wine and grape products improved EF in isolated arterial rat tissue.[317]

Most data support the idea that teas and the flavonoids they contain are able to improve NO bioavailability and thereby increase endothelium-dependent vasodilatation in healthy subjects.[318] A recent meta-analysis of intervention studies investigating the effects of teas on endothelial health in subjects free from or with pre-existing CVD risk factors supports the possibility that moderate consumption of tea enhances FMD.[319]

2.6.2.4 Micronutrients

Vitamins, including antioxidant vitamins A, C and E, and vitamin D have all been suggested to benefit vascular health.[181] Oxidative stress has a central role in the development of CVD and is proposed to affect EF through inflammation and reduced NO availability.[13] Therefore many studies investigating the effects of nutrients with antioxidant properties, including vitamins C and E and carotenoids, have been conducted to evaluate their role in CVD. There remains debate on the benefits of vitamin C supplementation on vascular function in healthy people and those with hypertension.[228, 320]

This review identified two cross-sectional studies[269, 276] and one intervention study investigating vitamin D.[258] Collectively these studies provide support for vitamin D supplementation in deficient individuals. The mechanism cannot clearly be deduced from the findings of the intervention study that lower concentrations of serum 25(OH)D (the inactive form of vitamin D) were associated with lower FMD. One proposed mechanism is that the association could be partly mediated by vascular endothelial cell conversion of inactive 25(OH)D to 1,25$(OH)_2$D, the active form. Reduced expression of vitamin D receptors and 1-α hydroxylase, the enzyme needed to convert inactive to active vitamin D, was found in endothelial cells of vitamin D-deficient subjects compared with vitamin D-sufficient subjects. A strong relationship between levels of 1-α hydroxylase and FMD, is consistent with this possibility. Reduced expression of vitamin D receptors and 1-α hydroxylase in vitamin D-deficient subjects may have limited the conversion of 25(OH)D to 1,25$(OH)_2$D, attenuated vitamin D signalling in the vascular endothelium and contributed to lower FMD.

There is insufficient evidence for a role of vitamin E in vascular health. Epidemiological evidence suggests a protective effect for vitamin E in plaque formation. In two large prospective studies, one in women and one in men, vitamin E intake, in the form of dietary supplements, was associated with decreased risk of CHD.[321, 322] Knekt (2004) found dietary vitamin E intake to be associated with reduced mortality from CHD in both men and women.[323] However, it is possible that the use of supplements was a marker of health conscious behaviour in these epidemiological studies.

The one intervention trial identified here found no benefit of vitamin E supplementation for PWV in post menopausal women.[251] In a randomized trial of a supplement containing 50 mg/d of α-tocopherol versus placebo in male Finnish smokers, supplementation failed to significantly affect the rate of ischemic heart disease mortality over the study period.[324] In the ARIC study, the use of dietary supplements accounted for some of the association of vitamin E with vessel wall thickness seen among the older women but not in the men. In fact, in the ARIC study, vessel wall thickness was greater in men taking vitamin E supplements compared with those not receiving vitamin E.

It is unclear why dietary supplements were related to less wall thickening in the older women but not the older men in ARIC. Possible sources of error including supplement use should be considered: 1) length of use was not ascertained; 2) frequency of use was not determined 3) appropriate dosage was not considered and 4) interactions with other foods and nutrients were not considered. Length of use is important because wall thickening occurs over a long time period. It is possible that fewer men were long-term users. Frequency of use gives a measure of compliance and is also important. It is possible that men may have been more intermittent users. Dosage is also important. For example, men being larger on average than women may have required larger doses. Interactions with other micronutrients might be relevant; for example, vitamin E may be absorbed more readily when taken with fat. Other components of foods rich in vitamin C and E may be the protective factor, rather than the vitamins themselves. Finally, other protective aspects of life-style may be associated with reduced wall thickness. In this case, differential effects of supplement use would be seen, for example, if the protective factor was correlated with vitamin use in women but not in men.

Few studies evaluated effects of lycopene, a potent antioxidant found in carotenoid compounds, on EF. In a study of healthy men with low fruit and vegetable (and therefore lycopene) intake, Kim and colleagues (2011) reported reductions in inflammatory markers in association with high doses (15mg/day) of lycopene given for 8 weeks.[244] Further studies are needed before findings can be generalised to wider populations, including females and individuals with higher fruit and vegetable consumption.

Overall, evidence for the use of micronutrients in protection of vascular health is lacking and most studies found no benefit for supplementation in healthy people. This is similar to conclusions drawn previously regarding the benefits of micronutrients for the treatment of hypertension. A large scale randomised primary prevention study including >5,000 healthy adults found that supplementation with antioxidants at nutritional doses had no effect on the risk of developing hypertension.[325]

2.7 Limitations

There are limitations to this review. Firstly, restricting the search to articles written in English may have led to omission of important findings. Secondly, due to heterogeneity of studies, comparisons between them were difficult. Carotid-femoral PWV is considered the gold standard measurement in assessment of arterial stiffness; however, investigators use a range of techniques in its measurement. Similarly, FMD is considered the gold standard for assessment of EF and only a small number of studies used this technique and RCTs are considered the most rigorous form of scientific evaluation but few have been undertaken in this area.

In many studies, interventions were short, ranging from 1 hour to 28 days, sample sizes were small and doses given and outcomes measured varied. Because diet in free-living humans is highly individual and influenced by many other factors it is difficult to randomise individuals to specific diets for time periods that may effect change and allow comparisons of effects of diets on outcomes. Furthermore, food and nutrients are metabolised differently according to individual characteristics.

2.8 Conclusions

A growing number of intervention studies have investigated short and long term effects of diet on endothelial health. Whilst acute studies that investigate short term effects of foods and nutrients on vascular structure and function are informative they cannot evaluate effects of habitual diet. To date, 33 prospective controlled trials including only 6 RCTs have been completed, and robust evidence for effects of diet on vascular health is lacking.

There is consistent evidence for increased risk of endothelial dysfunction with consumption of high fat meals. However, most studies are of short duration and evidence for effects of habitual high fat consumption on the vascular endothelium is scarce. There is some evidence to suggest that replacing a proportion of SFA with PUFA, particularly *n-3* LC-PUFA may offer benefits. However, further investigation in RCTs is required. To our knowledge, no RCT investigating

effects of *n-3* LC-PUFA on EF in healthy young individuals, who are free from CVD and have a low prevalence of risk factors, has yet been completed. In the absence of data from RCTs, evidence to underpin public health measures must be drawn from observational studies.

A small number of observational studies support an association of healthy diets with benefits for vascular health. One large scale epidemiological study suggests there are benefits for the endothelium in association with Mediterranean style diets.[295] However, Mediterranean style diets differ across countries and the current evidence is insufficient for firm conclusions to be drawn about specific diets. Most studies investigating effects of habitual diet on vascular structure and/or function used dietary pattern analysis. Longer term studies are difficult to conduct as adherence to prescribed diets by free living humans is unlikely. Furthermore, the period over which diet could affect measurable changes in endothelial structure and function is unknown.

To my knowledge, only six studies have investigated associations of dietary patterns with vascular structure or endothelial function. Four of these investigated relationships of dietary patterns with arterial stiffness.[268, 278, 281, 285] Two studies assessed EF but these were in older adults and no study measured FMD.[183, 287] Effects of dietary patterns on EF in healthy young people are not known, and further research is required.

Data from existing studies are insufficient to make public health recommendations for the role of specific diets and nutrients in primary prevention of atherosclerosis in healthy young people. Therefore, the present study investigates relationships between diet and endothelial dysfunction, the earliest indicator of atherosclerosis, in a group of healthy young adults, focussing on the role of *n-3* LC-PUFA and dietary patterns.

Chapter 3

Omega 3 Fatty Acids and Cardiovascular Disease

Fish oil is a whale of a story that not surprisingly gets bigger with every telling - Rogans, 1987

3.1 Introduction

Reports suggesting a relationship between fish intake and CVD risk began to emerge in the late 1970's, when observational studies among the Greenland Inuit people whose diet was based on fish found a lower incidence of CVD.[134, 135] This finding was subsequently extended to other high fish eating populations including Japanese,[192] South Pacific Islanders[193] and Alaskans.[326]

Increasing evidence now supports the hypothesis that fish is an important food and benefits for health, from high consumption of marine foods, can be attributed to their fatty acid content.[275, 327, 328] Epidemiological evidence and data from RCTs consistently report positive relationships between consumption of fish, (mostly at intakes providing about 1g combined eicosapentaenoic acid [EPA, C20:5 *n-3*] and docosahexaenoic acid [DHA, C22:6 *n-3*] per day), and reduced cardiovascular mortality and morbidity.[329-331]

3.2 Historical Background

Historically, fish was a valuable commodity for seafaring nations, providing both food and trade resources. In fact, during the middle ages, wealth and power in the North Sea region was largely dependent on the herring industry.[332] Rivalry over fishing rights has even led to wars between seafaring nations including the Dutch, French and English. The Battle of Herrings, in which the French requisitioned a fleet of ships delivering herring to the English army at Rouen, took place in 1429 and demonstrates the value of the targeted cargo.[333] Later, in 1703, competition for fishing rights led the French to undertake a further attack this time on a Dutch fishing fleet.[332]

By contrast, the 20th century "cod wars" occurring between Iceland and Britain through the 1950's and 70's were more sedate. However, disagreements over

fishing limits were sufficient to bring these two NATO allies to the brink of war. Such incidents serve to demonstrate the considerable value of fish both as a food and an economic resource.[332]

3.3 Health Benefits of *n-3* Fatty Acids

Benefits of marine foods are suggested to derive from their fatty acid content. Fatty acids were first described in 1929 by Burr and Burr from the United States.[334] However, it was not until 1937, when British physiologist Hugh Sinclair, proposed a relationship between deficiency of certain fatty acids and higher prevalence of Western disease, that possible benefits of fatty acids for long-term health were considered.[335] To investigate further Sinclair studied the Inuit people indigenous to Greenland. During his first visit in 1944 he noted that although the Inuit had the highest intake of fat in the world they did not suffer from the non-communicable diseases characteristic of Western societies. Moreover, he noted that the type of fatty acids habitually consumed differed between Inuit and Western populations; the Inuit diet being high in essential fatty acids. Subsequent analyses showed that the Inuit diets were richer in PUFA, the ratio to SFA was 0.84 as compared with 0.24 in Danes. PUFA were predominantly of the linolenic class (*n-3*) in Eskimos and the linoleic class (*n*-6) in Danes.[191]

Danish investigators, Bang (1976) and Dyerberg (1975) later confirmed a lower prevalence of CHD in the Inuit population and attributed this to their marine food based diet.[134 135] They also reported relationships between markers of CVD and a reduced incidence of heart attacks. Most lipid fractions were lower in the local Inuit population compared with both indigenous Caucasians and the migrant Inuit population living in Denmark leading to the conclusion that diet explained the almost complete absence of coronary atherosclerosis in indigenous Greenlanders.[135]

This hypothesis was supported by associations between changes in dietary habits such as fat consumption and disease.[336] Rapid expansion of populations in Europe, America and Australia created a need for more food. In the early 20th century rapid advances in food technology occurred as manufacturers strove to meet this need. The hydrogenation of fat was a major advancement allowing the

transformation of fats and oils to more stable forms that could be stored and transported more readily. This led to the introduction of saturated and *trans* fatty acids to the food market. At the same time, the decline in the herring industry reduced the availability of *n-3* fatty acids and changed the dietary fatty acid ratio in favour of *n*-6 fatty acids. Wide scale adoption of fat hydrogenation technologies and the demise of the herring industry were accompanied by a sharp rise in CHD.[336]

3.4 Nature of Fatty Acids

When Burr and Burr (1929) first described fatty acids they knew that they were essential to health but knew little about their structure, function or the mechanisms through which these benefits operated.[337] Numerous texts and articles now describe the structure, function and metabolism of fatty acids in detail, and are briefly summarized below in **Sections 3.4.1 - 3.4.2.**

3.4.1 Structure of Fatty Acids

Fatty acids are simple lipids comprising a straight chain of 6-24 carbon atoms each attached to one or two hydrogen atoms.[338] The hydrocarbon chain terminates in a hydrophilic carboxylic acid group at one end and a hydrophobic methyl group at the other. The terminal groups and the number of double bonds present in the hydrocarbon chain determine the lipids' structural and physiological properties **(Figure 3-1)**.

Saturated fatty acids contain no double bonds and therefore each carbon atom bonds with adjacent carbon, hydrogen or oxygen atoms. This allows a maximum number of hydrogen atoms in the fatty acid molecule which is termed 'saturated'. The presence of one or more double bonds in the chain reduces the number of hydrogen atoms and the molecule becomes unsaturated. Monounsaturated fatty acids contain one double bond and polyunsaturated fatty acids two or more.

The position of the first double bond in the carbon chain is indicated by the letter *n* counting from the methyl (CH_3) end **(Figure 3-1)**. For example, a fatty acid where the first double bond attaches to the 3rd carbon atom from the terminal

methyl group is termed *n-3*. Polyunsaturated fatty acids form two series with the first double bond occurring either at position 3 or 6 on the carbon chain. These are termed *n-3* and *n*-6 respectively.[338]

Double bonds allow the fatty acid to change its form (isomerism) to either a *cis* or *trans* configuration and this influences the molecular properties **(Figures 3-1a and b).** Most naturally occurring fatty acid double bonds are in the *cis* configuration a property that confers fluidity.

Figure 3-1 Structural representation of an *n-3* fatty acid

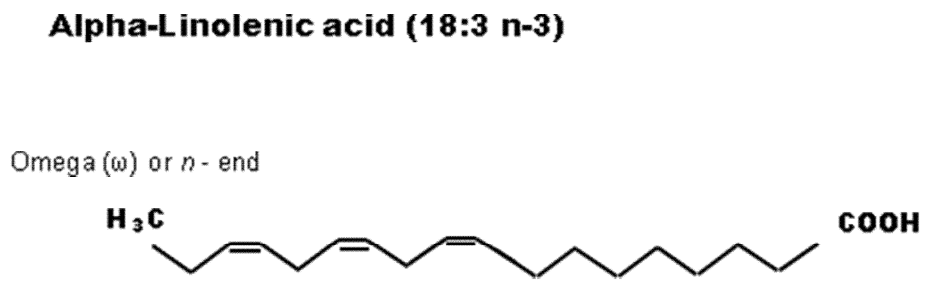


Figure 3-1a Structural representation of a *Cis* fatty acid

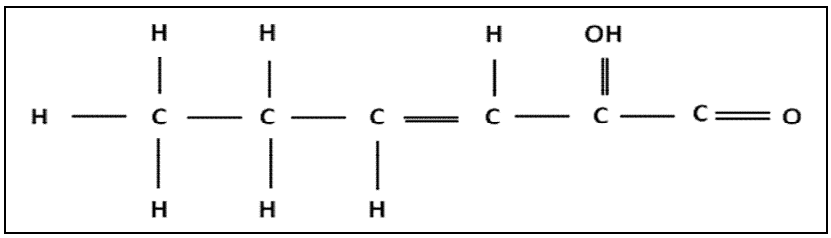
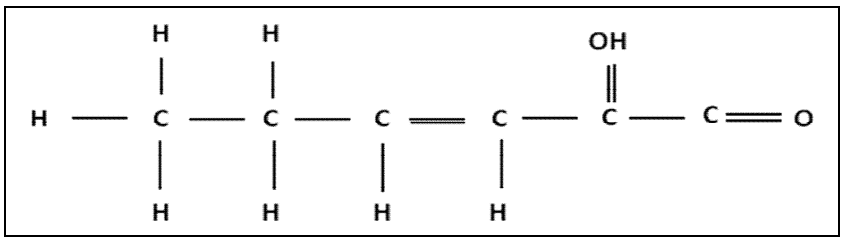


Figure 3-1b Structural representation of a *Trans* fatty acid



Fatty acids are found in plasma, either as free fatty acids (FFA), or attached to a glycerol backbone as part of a TG molecule **(Figure 3-2)**. The type of fatty acids

and their position on the TG molecule affects the molecule's properties. The position marked FA 1, 2 or 3 in figure 3-2 is also referred to as *sn* 1, 2 or 3. Fatty acids are also essential constituents of cell membrane phospholipids. Membrane lipids are essential in a number of biological functions from membrane trafficking to signal conduction.[338, 339] A bipolar lipid bilayer provides a semi permeable barrier allowing compartmentalisation within cells and separation of ions and solutes **(Figure 3-3)**. This enables specialised functions of organelles and maintains cell membrane electric potentials.[340]

Figure 3-2 Triglyceride Structure

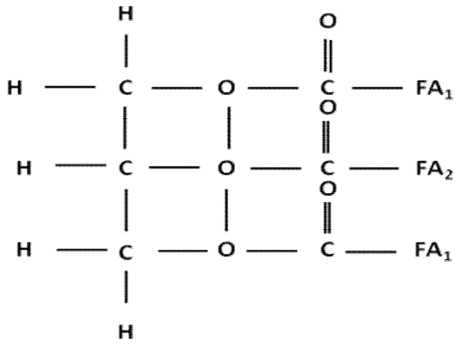
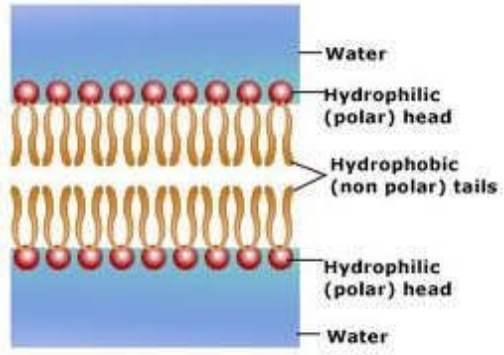


Figure 3-3 Lipid bilayer (source: Google Images,TutorVista.com)



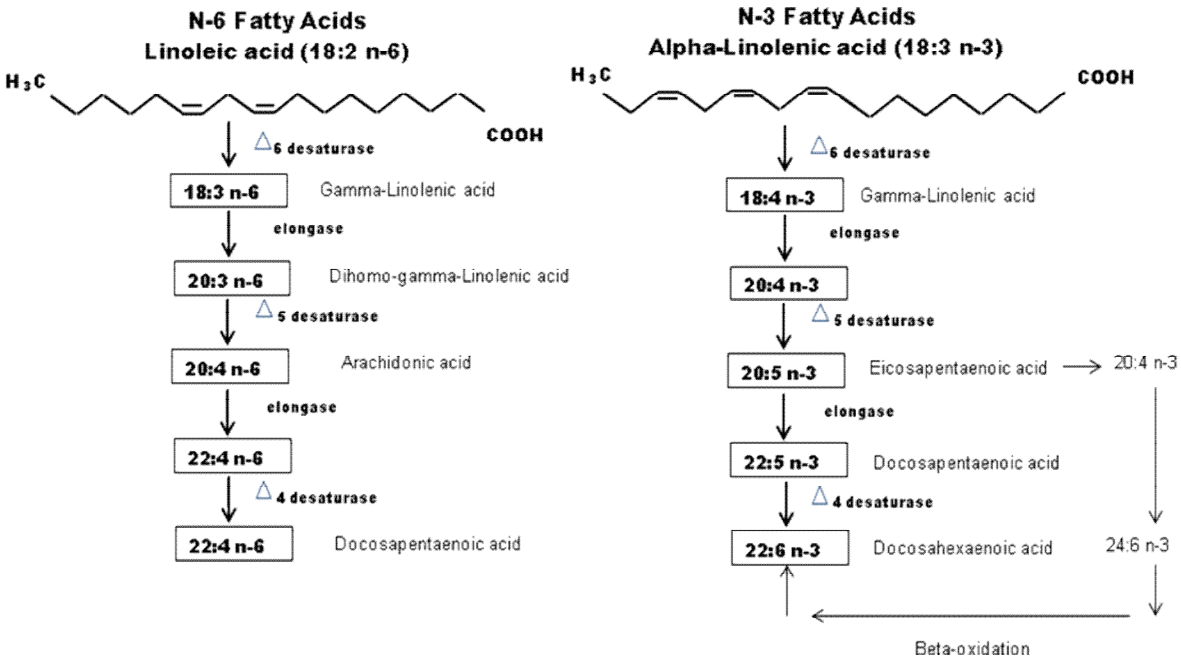
3.4.2 Essentiality of Fatty Acids

Mammals lack an enzyme needed to insert double bonds between adjacent carbon atoms at positions 1-6 of the hydrocarbon chain.[341] **(Figure 3-1)**. Therefore, there is a dietary requirement for linoleic acid (LA, C18:2 *n*-6) and alpha linolenic acid (ALA, C18:3 *n-3*), essential fatty acids (EFA) of the omega-6 and omega-3 series respectively.

LC-PUFAs are produced through a process of desaturation and elongation of fatty acid hydrocarbon chains (Figure. 3-4).[343] The dietary requirement for EFA's is relatively small but nevertheless important. In the UK it is recommended that LA should provide at least 1% and ALA at least 0.2% of total daily energy intake.[159]

Biologically important fatty acids include arachidonic acid (AA, C20:4 n-6), derived from LA, and EPA and DHA formed from the parent *n-3* fatty acid, ALA. These have important physiological roles, for example, both AA and EPA are precursors to eicosanoids, chemicals with multiple functions in a number of important processes including mediation of chemical reactions and regulation of inflammation **(Figure3-5)**. [343]

Figure 3-4: Formation of Long Chain Polyunsaturated Fatty Acids (Adapted from De Caterina *et al.*,(2001)) [343]



3.4.3 Metabolism of Fatty Acids

The parent fatty acids of both the *n*-6 and *n-3* series are derived from plant oils. High sources of ALA include flaxseed, rapeseed, soya and some nuts e.g.

walnuts and almonds. Sunflower and corn oils are the predominant sources of LA in Western diets. LC-PUFA are formed from the parent fatty acids or taken in directly from dietary sources. *n-3* LC-PUFA are found in marine based plants and micro-organisms such as phytoplankton, seaweeds and algae or from animals that feed directly on plant sources (e.g. fish and poultry) **(Box 1)**. Metabolism of LC-PUFA results in the production of eicosanoids, regulatory compounds that have direct effects on human health.[344]

3.4.3.1 Eicosanoids

Eicosanoids are formed from non-esterified or free fatty acids (FFA) that must therefore be in constant supply. A pool of LC-PUFA is maintained from dietary sources and through hydrolysis of storage material, for example from cell membrane phospholipids.

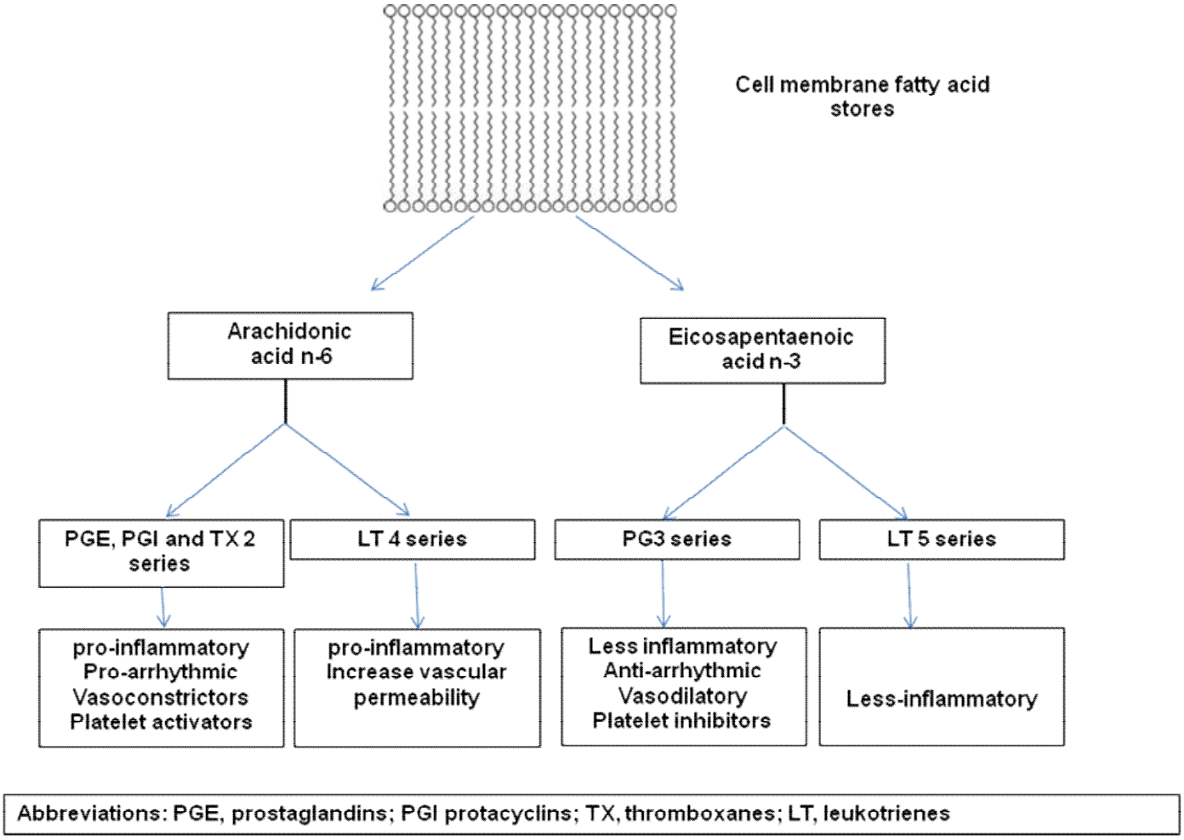
Dietary LC-PUFA are directed through one of three metabolic pathways and regulated by enzyme availability. The acyl-COA pathway regulates oxidation and storage, the cycloxygenase and lipoxygenase pathways provide prostaglandins, thromboxanes, prostacyclins and leukotrienes respectively. Fatty acids compete for enzymes in all pathways and the balance of eicosanoids produced depends on the availability of fatty acids **(Figure 3-5)**.{De Caterina R., 2001 3498 /id}

Eicosanoids are implicated in many disease processes, primarily through their role as mediators of the inflammatory response.[346, 347] Eicosanoids produced from *n*-6 fatty acids are generally pro-inflammatory compared with those from *n-3* fatty acids. Therefore diet, as a provider of fatty acid substrates, is an important regulator of eicosnaoid production.

Eicosanoids form four distinct families: prostaglandins, protacyclins, thromboxanes and leukotrienes **(Figure 3-5)**. Each family further divides into separate series. Different activities within series help explain the health effects of *n-3* and *n*-6 fatty acids.[348] High dietary intake of *n*-6 fatty acids leads to a predominance of arachidonic acid (AA) in cell membrane phospholipid. In this instance AA is the major substrate for eicosanoid production and pro-inflammatory mediators are favoured. Diets higher in *n-3* fatty acids allow partial

replacement of AA acid by EPA and a less inflammatory eicosanoid profile results.

Figure 3-5 Eicosanoid metabolism and function



3.5 Cardioprotective Effects of *n-3* Fatty Acids

Various mechanisms through which *n-3* LC-PUFA exert beneficial effects on health have been proposed. Several large scale human studies have reported benefits for a range of CVD risk factors including prevention of arrhythmia,[349] decreased platelet aggregation,[350, 351] improvements in endothelial function,[352, 353] reductions in BP,[354, 355] plasma TG,[356, 357] and inflammatory response,[358] as well as inhibition of platelet activation and other anti-atherosclerotic effects.[262, 359]

3.5.1 *n-3* LC-PUFA and CVD Risk Reduction

Benefits of *n-3* LC-PUFA from fish and fish oil supplements have been shown for both primary and secondary prevention of CVD. Many epidemiological studies

have provided evidence for relationships between fish intake or *n-3* FA status and reduced risk of CHD and CVD outcomes.[360-365] The relationship is strongest for fatal heart attacks. For instance, Albert and colleagues (1998) reported from the US Physicians Health Study that higher consumption of fish was associated with a lower risk of sudden cardiac death in men (Relative Risk (RR) = 0.48; 95% confidence interval (CI): 0.24 - 0.96 [*P* = 0.04] for once weekly compared with once monthly consumption).[361] Lemaitre (2003) also reported a decreased risk of fatal CHD for older adults in association with *n-3* fatty acid status (RR = 0.32; 95% CI: 0.13 - 0.78 [*P* = 0.01]).[363] This strong association with fatal CHD suggests anti-arrhythmic properties of *n-3* LC-PUFA are likely to confer protection **(Section 3.6.2)**.

More recently a large prospective cohort study including 25,573 men and 28,653 women reported decreased risk of acute coronary syndrome for men consuming the highest quantities of fatty fish.[365] A 33% reduction in risk was found for men in the highest quintile of fatty fish intake (>6g/day) compared with those in the lowest quintile. A lower risk was found for women raising the possibility of gender differences regarding benefits of fish consumption.

Long-term fish intake may be more beneficial than short term intake. The Zutphen study, conducted among a large cohort of older men, reported an inverse association between long term fish intake and sudden coronary death (Hazard Ratio (HR) = 0.46; 0.27, 0.78) for long term intake compared with recent consumption.[364] The effect was most marked in the younger age group (40-50 years) and declined between age 50 (HR = 0.32; 95% CI: 0.13 - 0.80) and 80 years (HR = 1.34; 95% CI: 0.58 - 3.12). Benefits were seen at relatively low *n-3* intakes of around 250mg EPA and DHA combined daily that offered greater protection compared with no intake. No further benefit was associated with intakes greater than 250mg/day, suggesting a threshold around this level of intake.

3.5.2 Recommendations for LC-PUFA Intake

Adults in the UK consume on average 10 and 11g per day of oily fish for men and women respectively.[160] This would provide approximately 150mg per week *n-3*

LC-PUFA depending on the type of fish **(Box 2)**. Current advice from the UK Scientific Advisory Committee for Nutrition (SACN) is to consume two portions of fish per week, one of which should be oily.[366] Based on an average 100g portion, this would provide approximately 450mg/day *n-3* LC-PUFA. Concentrations of *n-3* LC-PUFA are highest in fish, particularly oily fish, although some plant sources are available.

Box 2: Dietary sources of *n-3* Fatty Acids

Sources of *n-3* Alpha Linolenic Acid	**Per 100g (g)**
Rapeseed oil	9.6
Soya oil	7.3
Flaxseed oil	22.8
Walnuts	7.5
Almonds	0.27
Sources of *n-3* LC-PUFA	
Oily Fish	
Herring – fresh, frozen or preserved	1.8
Mackerel – fresh or frozen	2.8
Kippers – fresh, frozen or canned	3.5
Pilchards – canned	3.0
Tuna – fresh or frozen	1.3
Trout –fresh or frozen	1.3
Sardines – fresh or canned	2.3
Sild or skippers – canned	2.7
Salmon – fresh or canned	1.9
Shellfish	
Crab (fresh)	1.1
Mussels	0.7
Prawns	0.1
White Fish	
Cod	0.26
Haddock	0.17
Plaice	0.32
White Meat	
Chicken	0.1-0.33
Turkey	0.3
Liver	0.1
Enriched Foods	
Eggs	0.35 (per egg)
Milk	0.25-0.4
Bread	0.47

Source: Fatty Acids: 7th supplement to the 5th edition of McCance and Widdowson's The Composition of Food Ministry of Agriculture Fishery and Foods, 1998.[367]

3.6 *n-3* LC-PUFA and secondary prevention of CVD

The strongest evidence for a protective role of *n-3* LC-PUFA is for secondary prevention of CHD. Data from epidemiological studies, RCTs and meta-analyses provide support for this hypothesis.

3.6.1 Dietary interventions in secondary prevention of CVD

The first clinical trial in humans investigating *n-3* LC-PUFA effects on CVD was The Diet and Re-infarction Trial (DART).[195] Burr and colleagues performed this RCT in a large group of men with a previous heart attack (*n* = 2,033). Participants were randomised to receive different types of dietary advice: *(1)* to reduce fat intake and increase the ratio of polyunsaturated to saturated fat, *(2)* to increase oily fish, or *(3)* to increase cereal fibre intake or to a control group that received no dietary advice. There were no reported benefits from the fat or fibre advice. However, a 29% reduction in all cause mortality occurred in 1,015 men advised to eat two large portions of oily fish per week, (providing about 200 to 400mg *n-3* LC-PUFA daily in total), when compared with the 1,018 men who had not received such advice. This difference was entirely attributable to a reduction in deaths from heart disease. Of note, the incidence of heart attacks was not lower and this suggests the reduced mortality was due to anti-arrhythmic properties of *n-3* LC-PUFA **(Section 3.6.2)**.

Since DART, numerous trials have investigated effects of *n-3* LC-PUFA on secondary prevention of CHD. The most notable include the GISSI Prevenzione [196] and JELIS studies.[132] In the GISSI study participants with a previous MI were randomised either to receive: (*1*) supplements of *n-3* LC-PUFA (1g daily, *n* = 2,836), (*2*) vitamin E (300mg daily, *n* = 2,830), (*3*) both LC-PUFA and vitamin E (*n* = 2,830), or (*4*) no supplementation (control, *n* = 2,828) for 3.5 years. Treatment with *n-3* LC-PUFA significantly reduced all cause mortality (relative risk (RR) 0.59; 95% CI: 0.36 - 0.97 [*P* = 0.037]) and was largely attributed to a decrease in sudden death (RR 0.47; 95% CI: 0.219 - 0.995 [*P* = 0.048]).[196]

The JELIS study added further support for promoting consumption of LC-PUFA. 18,645 Japanese patients with a history of CAD and TC ≥6.5mmol/L were

randomised to receive either 1.8g/d EPA with cholesterol lowering medication (statin) (*n* = 9,326) or to statin only (control; *n* = 9,319). Patients were followed up to 5 years and fewer coronary events were reported in the intervention group compared with controls (intervention: 262 (2.8%), control: 324 (3.5%) [*P* = 0.011]).[132]

Overall, the most important effect of *n-3* fatty acids in secondary prevention is a reduction in mortality following MI. These effects are proposed to operate primarily through anti-arrhythmic actions.

3.6.2 Anti- arrhythmic effects

More than half of all deaths from CHD are sudden, occurring within one hour of symptoms of an acute MI. These are attributed to sustained ventricular arrhythmias.[349] Gudbjarnason and Hallgrimsson (1976) were the first to suggest anti arrhythmic properties of LC-PUFA following investigations in animal models.[368] In these experiments, dietary fat was manipulated in rats. Saturated fat was replaced by either MUFA or LC-PUFA for 3 months, after which ventricular fibrillation was induced. Fatal ventricular fibrillation was significantly reduced by both *n*-6 (Fatalities: 2/25(8%) and *n-3* (fatalities: 0) PUFA but not by MUFA (fatalities: 9/25 (36%) or saturated fatty acids (SFA) (fatalities: 10/24 (46%).[369]

Anti-arrhythmic actions of *n-3* LC-PUFA were further investigated in non-human primates. Marmoset monkeys fed diets supplemented with *n-3* LC-PUFA-rich tuna fish oil for 24 months had significantly elevated mean ventricular fibrillation threshold compared with those fed a diet supplemented with saturated animal fat or a reference diet not supplemented with fat. Dietary tuna fish oil was associated with a low incidence of sustained fibrillation episodes and an absence of fatalities.[370] Studies have been criticized because of possible confounding factors (e.g., by effects from non-fat dietary components, physical activity and other lifestyle factors) occurring in long-term dietary studies in animals.

Mechanistic studies provide support for these findings.[308, 371-373] However, short-term studies such as these, based on acute ingestion of LC-PUFA, cause

increases in plasma concentrations of *n-3* LC-PUFA that may not be achievable in free living subjects. Studies in free living human subjects are therefore needed to demonstrate biological effects of *n-3* LC-PUFA.

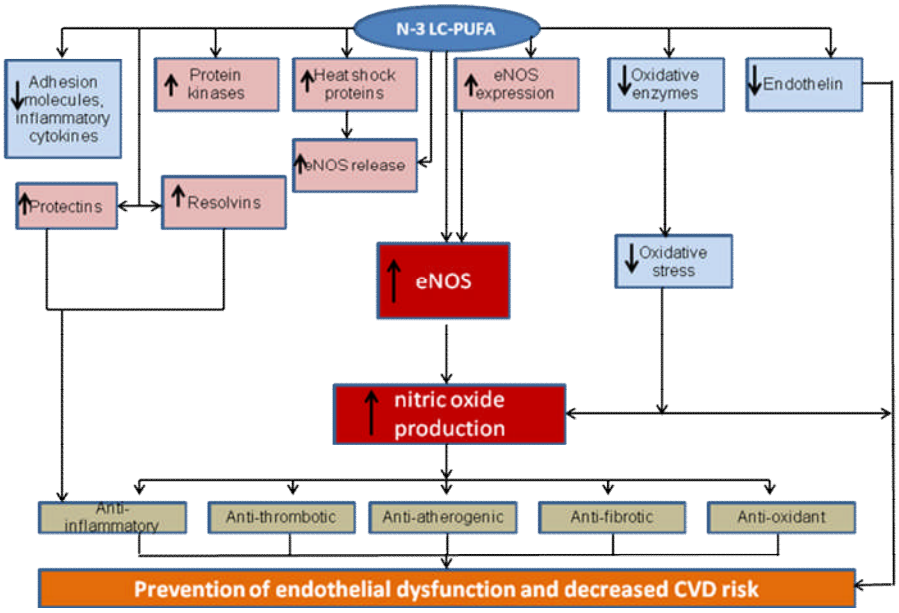
3.6.2.1 Mechanisms for anti-arrhythmic effects of *n*-3 Fatty Acids

Proposed mechanisms for anti-arrhythmic effects of *n-3* LC-PUFA include actions to reduce excitability of cardiomyocytes (heart cells).[374] Evidence comes from studies in animal models[374-376] where *n-3* fatty acids were reported to modulate ionic currents in rat cardiomyocytes.[377] Specifically, they have been shown to inhibit fast sodium[377-379] and calcium currents[380] responsible for rapid depolarisation in cell membranes during MI. Rapid depolarisation makes heart muscle more excitable and induces arrhythmia. *n-3* LC-PUFAs favour polarization and decrease the risk of fatal arrhythmias.[349]

3.7 *n-3* LC-PUFA and primary prevention of CVD

Several studies have reported cardioprotective effects of *n-3* LC-PUFA.[132, 327, 381-384] These have largely been attributed to lipid-lowering actions that are well established.[299] Additionally, anti-inflammatory, antioxidant and anti-thrombotic actions could also have major roles.[385] The vascular endothelium has a major role in many CVDs and dysfunction is an early indicator of atherosclerosis. Endothelial dysfunction occurs as a result of reduced NO and increased oxidative stress **(Figure 3-6)**. As reviewed in **Chapter 2**, many studies have demonstrated a protective role of *n-3* LC-PUFA in primary prevention of CVD by improving endothelial function. A large body of evidence also supports protective effects of *n-3* LC-PUFA for classical CVD risk factors. Evidence and potential mechanisms are discussed below.

Figure 3-6 Proposed mechanisms for a protective role of *n-3* LC-PUFA in endothelial dysfunction



Adapted from Balakumar and Tanja (2012).[385]

3.7.1 *n-3* LC-PUFA and hypertension

Clinical trials and 3 meta-analyses, involving subjects with and without HT, dyslipidaemic patients, diabetics and elderly subjects confirm the hypothesis that *n-3* LC-PUFAs are able to slightly, but significantly reduce BP.[386] Two meta-analyses independently concluded that *n-3* LC-PUFA reduces BP in a dose dependant manner (-0.66/0.35mmHg/g *n-3* LC-PUFA) at intakes ≥3g/d.[387, 388]

Antihypertensive effects of fish oils have mainly been achieved at relatively large doses (4-15g/day) with *n-3* LC-PUFA provided as a dietary supplement.[389] [390-392] Dietary *n-3* LC-PUFA has also been reported to benefit BP. However, interventions provided high intakes that were not representative of usual diet. For example, daily fish meals that provided 3-4g/day of *n-3* fatty acids reduced BP in overweight patients receiving treatment for hypertension.[393]

There are few published reports of studies among healthy subjects free from HT. One study reported no benefits of *n-3* LC-PUFA for HT.[394] However, interpretation may be hampered by the design of this trial. Participants were first randomised to diets rich in either SFA or MUFA then after 3 months, were further

randomised to *n-3* LC-PUFA or placebo. Differences in background diet were apparent between groups. For example, higher dietary fibre and lower cholesterol were observed in the MUFA diet group. These and other dietary factors are known to influence BP, and it was not possible to exclude effects of multiple dietary exposures on BP in this trial.

A recent meta-analysis of RCTs investigating anti-hypertensive effects of *n-3* LC-PUFA concluded that high intake of fish oil reduced BP. Effects were larger in older populations (>45 years) and in those with pre-existing HT.[355] The dose was high in most trials (median dose: 3.7g/day) and effect sizes moderate: fish oil reduced SBP by 2.1mm Hg (95% CI: 1.0 - 3.2 [$P < 0.01$]) and DBP by 1.6mm Hg (95% CI: 1.0 - 2.2 [$P < 0.01$]). Effects of *n-3* LC-PUFA were attenuated in double-blind trials and where sample size was larger suggesting trials with smaller sample size may be subject to publication bias.

3.7.1.1 Mechanisms for anti-hypertensive effects

The physiological mechanisms by which *n-3* fatty acids may reduce BP are not well-established. Explanations include reductions in heart rate (HR) and vascular resistance with high *n-3* LC-PUFA intake.[354] A decrease in HR is reported in most studies investigating cardiovascular benefits of *n-3* fatty acids.[395] DHA appears to have the strongest effect.[388] *n-3* LC-PUFA also reduces angiotensin-converting enzyme activity, angiotensin II formation and activates the parasympathetic nervous system leading to increased arterial compliance of both small and large arteries.

3.7.2. *n-3* LC-PUFA and the Lipid Profile

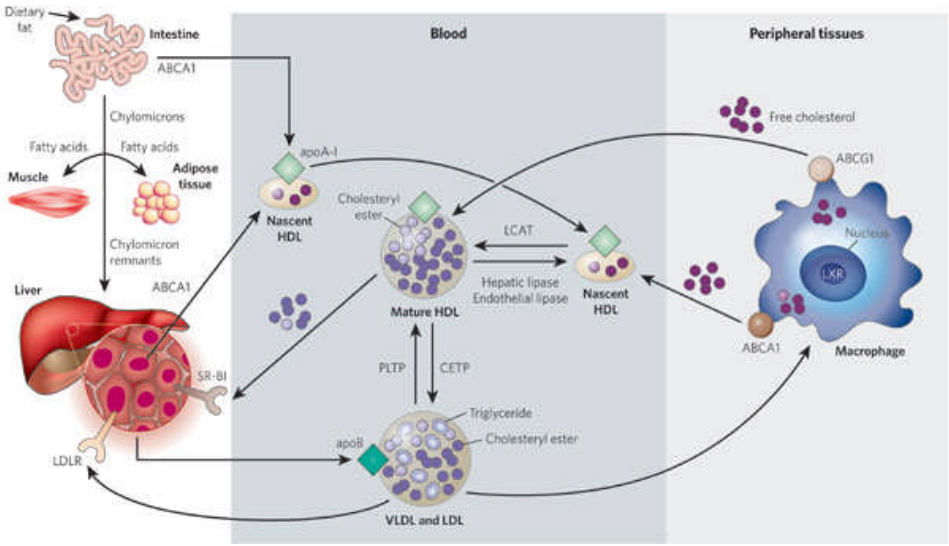
In 1948, the rising burden of CVD in the US prompted the initiation of the Framingham project; the initial aim of which was to identify risk factors for CHD **(Chapter 1, Section 1.5.2.3)**. Raised blood cholesterol was identified as a major risk factor and a strong correlation was seen between increasing CV events and rising TG.[104, 396, 397]

Lipoprotein sub-particles were also found to be influential. For example, low levels of HDL cholesterol were as much a risk factor for CHD as high LDL cholesterol. Data from Framingham suggest that high TG level is a risk factor for CAD particularly when plasma LDL to HDL cholesterol ratio is greater greater than 5.0. Furthermore, TG rich lipoproteins varied in their propensity to atherogenicity **(Section 3.7.3.3).** Evidence from observational and experimental studies provides strong support for a relationship between TG and CVD.[398, 399, 400] These are discussed in detail below in **(Section 3.7.3)**. Lipoprotein metabolism is central to the understanding of its role in CVD. Therefore an overview is provided in the following section.

3.7.3 Lipoprotein Metabolism

Lipoproteins are metabolised via two biochemical pathways. The exogenous route deals with dietary lipids whereas hepatic lipoproteins are processed via the endogenous pathway.

Fig. 3-7 Lipoprotein Metabolism (Google images, nature.com)



3.7.3.1 Exogenous Pathway

The small intestine absorbs dietary lipids for example from TG, phospholipids, and cholesterol. These combine with apolipoprotein (apo) B-48 to form chylomicrons (CM). Newly formed (nascent) CM are secreted into lymphatic vessels from where they pass via the thoracic duct into the bloodstream.

Nascent CM accept apo from HDL particles to form mature HDL. CM activate lipoprotein lipase (LPL), an enzyme which catalyzes the hydrolysis of TG to glycerol and FFAs for use in respiration or for storage **(**see **Figure 3-2** for TG structure**)**. Once hydrolyzed, CM become remnant particles which continue circulating until they interact, via apoE, with specific remnant receptors found mainly in the liver. CM transport TG to the liver, VLDL transports newly synthesised TG from the liver to adipose tissue, LDL transports cholesterol from the liver to the tissues and HDL returns cholesterol from the tissues to the liver **(Figure 3-7)**.

3.7.3.2 Endogenous Pathway

The liver is an important source of lipoproteins, principally VLDL. TG and cholesterol combine with apoB-100 to form nascent VLDL particles. Lipoproteins apoC-II and apo-E, acquired from HDL, are required to form mature VLDL. Mature VLDL particles circulate and are hydrolysed by LPL to release glycerol and FFA. Hydrolyzed VLDL particles become VLDL remnants that circulate and are eventually absorbed by the liver in an interaction involving apo-E and remnant receptors. Further hydrolysis may take place in the liver through the action of hepatic lipase. This releases glycerol and FFA, leaving behind LDL. LDL particles are cholesterol rich and can be absorbed by the liver and peripheral cells. LDL binds to specific (LDL) receptors on target tissues via apoB-100 or apo-E. Elevated lipoproteins increase the risk of CHD. In particular lipoprotein a (Lpa), remnant lipoprotein particles are implicated. Dietary fats have an important role in CHD risk through their influence on plasma lipoprotein concentrations.

3.7.3.3 Atherogenicity of Lipoprotein Particles

Atherogenic properties of lipoproteins are determined by a number of factors. Size influences their likelihood of entry to the sub-endothelial space and small particles are more readily taken up. ApoB has a high affinity for attachment to the sub-endothelial matrix and does this via a vast network of specialised molecules (proteoglycans) on its surface. Susceptibility to oxidation is also important. Modification by oxygen of apoB makes it more palatable to macrophages so encourages greater uptake and subsequent foam cell formation.[401]

Atherogenic lipoproteins such as CM and VLDL remnants are generated during post-prandial lipaemia (PPL) – a non-fasting state following a meal. Extended or frequent PPL increases the risk of oxidative modification of LDL and the production of small-dense LDL particles which are widely considered to be the most atherogenic lipoprotein particles. Prolonged or frequent PPL increases TG flux through plasma thereby raising the likelihood of oxidative modification of lipoprotein particles.

3.7.4 Effects of *n-3* LC-PUFA on the lipid profile

The fatty acid content and profile of the diet influences PPL. Diets high in SFA increase total and LDL cholesterol and stimulate transfer of cholesterol to VLDL and CM remnants. MUFA appear to have little effect on PPL itself but may reduce apoB-48 and circulating remnant particles.[402] *n-3* LC-PUFA reduce both fasting and postprandial TG levels.

Fish oils have repeatedly been shown to reduce plasma TG in a dose-dependent manner.[357] Early investigations used preparations containing both EPA and DHA as reviewed in Harris.[356] Both EPA and DHA reduced serum TG with the greatest effects seen in patients with raised serum TG (hypertriglyceridaemia - HTG).[403] Both parallel (n = 29) and crossover (n = 36) studies have drawn the same conclusions: TC was not affected by *n-3* consumption, LDL cholesterol concentrations rose by 5-10% and HDL cholesterol by 1-3%. The greatest benefit was seen for TG concentrations which decreased by 25-30%. In early studies, benefits of *n-3* LC-PUFA were mainly attributed to EPA, an assumption based on the fact that the only commercially available source of fish oil, Menhaden oil, contained more EPA than DHA (18% *vs* 9%).[299] All these trials used supplements containing large doses of fish oil (2-5g/day combined EPA and DHA (EPA/DHA).

Recently, Sanders (2011)[253] supplemented a group of healthy older individuals (aged 45–70 years) with lower doses of combined EPA and DHA (0.4–1.8g/day) for a period of 12 months. A reduction of 16% in TG concentration was reported for the group receiving the highest level of supplementation (P for trend 0.014).

Based on results from early trials, researchers focussed mainly on supplements combining EPA and DHA. However towards the late 1990s studies investigating separate effects of EPA and DHA were initiated. It is currently uncertain whether EPA or DHA or both together are responsible for the protective effect. One trial among healthy subjects reported only a small benefit of EPA supplementation on TG reduction.[404] However, this study provided a much lower dose EPA (1.8g/day) than previous studies **(Table 3-1)**.

DHA can be synthesised from ALA (C18:3 *n-3*) in mammals. However, its formation is limited as there is competition with LA for enzymes central to desaturation and elongation processes[405] **(Figure. 3-4)**. Studies investigating effects of single preparation DHA mostly used supplements providing about 1.6g/day.[406-412] Two studies used slightly higher doses 2-3g/day)[412, 413] and achieved similar reductions in TG **(Table 3-1)**.

Most studies investigating effects of *n-3* on the lipid profile included small sample sizes (*n* = 13–60) and interventions varied in duration from 1 to 12 months. However, collectively these studies provide support for a beneficial effect of *n-3* LC-PUFA on TG, achieving reductions in the range of 5-25% in healthy individuals and between 15-50% in individuals with pre-existing dyslipidaemia. Effects on other lipid fractions, however, are not so clear. Some studies report beneficial effects on HDL cholesterol whereas others have found no such effect. Some studies have reported increases in total and LDL cholesterol **(Table 3-1)**.

3.7.4.1 Effects of combined EPA and DHA

Beneficial effects of *n-3* LC-PUFA on the lipid profile are most pronounced among people with higher CVD risk. In one example twenty-two patients with pre-existing CAD were given 3.4g/ day of EPA/DHA for 4 weeks. Serum TG was reduced by 17% and HDL was unchanged.[414] Effects are even greater in individuals with HTG.

Thirteen HTG patients were supplemented with either a concentrated ester of combined EPA and DHA or with a TG-rich fish oil for twelve weeks in a randomized crossover design intervention study. Doses ranged from 2-4g/day

and both oils reduced TG by 50%. LDL cholesterol increased by 18% and apo-B by 23%.[415] A similar reduction was reported in patients with severe HTG (500–2000mg/dL). In forty patients supplemented with 3.4g/day EPA and DHA for four months TG reduced by 45% compared with patients randomized to placebo.[416] The large reduction in TG could be due either to the longer supplementation period or to a greater effect in individuals with more severe HTG.

n-3 dose in most studies equated to a pharmaceutical dose rather than a level usually seen in the diet. In a randomized study in 28 HTG patients comparing 3.4g/day Omacor (providing 1.88g EPA and 1.48g DHA) (Omacor, Pronova Biocare, Oslo, Norway) with Gemfibrozil (Pfizer, USA), a fibrate preparation of the treatment of HTG, both treatments reduced TG (Omacor 39%, Gemfibrozil 42%). Both HDL and LDL cholesterol concentrations increased as a result of the intervention.[417]

Increases in LDL cholesterol are reported from many *n-3* supplementation studies [418-420] which is of potential concern. For example, in one randomized study among 33 men with raised cholesterol (hypercholesterolemia [HC]), supplementation with 3.4g/day EPA and DHA for 6 weeks, reduced TG greatly (by 39%) however, a concomitant increase in LDL cholesterol (9%) was also reported.[418] Similarly, Calabresi and colleagues reported that supplementation with Omacor reduced plasma TG by 27-44% and although TC did not change LDL increased by 20-25%.[419, 420]

Increases in HDL cholesterol are reported in some studies and this may be beneficial **(Table 3-1)**. For example in a randomized, double blind study of 43 hypertensive patients 3.4g/day Omacor reduced TG by 21% and increased HDL by 5% ($P < 0.01$). No significant changes in total or LDL cholesterol were observed.[391]

Findings from larger studies support those from the smaller studies reviewed here. In a randomized trial of 10 week's duration Bonaa (1992) compared 5.1g/day fish oil containing EPA and DHA with 6g/day corn oil in 156 subjects. A 21% reduction in TG was reported for subjects randomised to fish oil.[390] In a 6

month randomized study of 935 individuals with HTG, 1.7g/day EPA and DHA also reduced TG by on average 21% and this time using a lower dose of fish oil.[421] A concomitant increase in HDL cholesterol was also reported.

Increasing the intervention period by 1-2 years resulted in a further monthly decrease in TG for individuals supplemented with *n-3* LC-PUFA and an increase in controls supplemented with corn oil (intervention: -1.3%, control*:* +0.35% [*P* < 0.001]). HDL cholesterol increased in both groups but to a greater extent in the intervention group (intervention: 1.1%; control: +0.55% [*P* = 0.002]). It is currently uncertain whether a longer duration of supplementation may have greater long term benefits. Furthermore adherence to such long-term interventions could prove difficult.

3.7.4.2 Separate Effects of EPA and DHA

The relative contributions of EPA and DHA to cardiovascular health remain to be elucidated. Investigations into independent effects of EPA and DHA have been hampered by a lack of availability of purified extracts of individual fatty acids.[299] Data from RCTs have only recently begun to emerge - reviewed most recently by Mori & Woodman (2006)[422] and Ryan (2009).[299] Studies investigating both combined and independent effects of EPA and DHA have been conducted in a range of subjects including healthy,[253, 406-410, 423-426] hypertensive,[427] dyslipidaemic,[428, 429] and overweight individuals.[430] **(Table 3-1)** The individual effects of EPA and DHA are considered below.

3.7.4.3 Controlled trials with EPA

To date five controlled studies have investigated separate effects of EPA on serum lipids and lipoproteins. These studies report reductions in TG of between 5 and 21%.[404,425, 427-428, 429] In most trials, supplements provided about 4g/day EPA. One trial provided a lower dose (1.8g/day) and this proved ineffective in lowering TG in patients with angina.[404] Control groups were either given placebo oils including olive oil,[427, 429] or corn oil[425] or received no intervention.

3.7.4.5 Controlled trials with DHA

Twelve controlled trials examined effects of DHA on the lipid profile in adults.[406-413, 431-434] A dose of approximately 1.5–1.8g DHA/day was used in most trials. DHA reduced TG by about 12-24% in 9/12 trials. Only two trials reported substantially smaller or no reduction in TG. One trial conducted among children with familial hyperlipidaemia reduced TG by only 5%[434] and the earliest trial by Conquer and colleagues (1996) reported no change in TG where doses between 0.75 and 1.5g/day were given.[407] Hamazaki and colleagues (1996) observed no benefits for TG concentration where dosage was based on bodyweight (10 capsules for ≤50kg, 11 capsules for >50kg but <55kg and 12 capsules for >55kg). Larger decreases in TG occurred in patients with pre-existing dyslipidaemia.[410] However, Geppert and colleagues (2006) supplemented healthy vegetarians with a lower dose (0.9g/day) and still reported a mean reduction in TG of 23%.[432]

3.7.4.6 Effects of n-3 LC-PUFA on Lipoprotein sub-particles

LDL cholesterol increased in a number of supplementation studies either with EPA and DHA combined preparations[417-419, 435] or DHA alone[407, 409, 411-413, 428, 429, 432, 434] but in only one trial supplementing with EPA only.[404]

An increase in LDL particle size following DHA supplementation was reported in one study among diabetic subjects.[427] This may be important because LDL particle size is an important risk factor for CVD. Dyslipidemia is seen in association with T2DM and is marked by an accumulation of small dense LDL particles in plasma. These are more likely to form potentially harmful compounds by reacting with sugars (glycation) or oxygen (oxidation) and may contribute to endothelial dysfunction also seen in T2DM. Small dense LDL is also associated with increased CCA-IMT, a recognised risk factor for atherosclerosis.[436]

Very low density lipoprotein cholesterol (VLDL) is a particularly atherogenic lipoprotein.[90, 437] Several studies have reported reductions in VLDL in association with *n-3* supplementation.[417-419, 428, 438] Studies supplementing with combined EPA and DHA preparations have reported reductions in VLDL of 20-65%.

HDL cholesterol increased by between 8 and 15% in 4 studies using combined EPA and DHA preparations,[391, 420, 421, 439] Smaller increases of 5 and 7% respectively were reported in two studies using EPA alone.[404, 428] The most consistent benefits for HDL cholesterol are reported in association with DHA supplementation where 9/12 studies reported increases ranging from 5-17%.[406-408, 411-413, 432-434] No significant differences in serum lipid concentrations were reported at baseline in any trials. Beneficial effects on lipoprotein sub-fractions were reported in some studies. For example, HDL2 increased by 12-37% in three studies[407, 427, 429] – the greatest increase was seen in dyslipidaemic subjects.[429]

3.7.4.7 Mechanisms for effects of n-3 LC-PUFA on lipoproteins

Reductions in plasma TG seen in association with *n-3* LC-PUFA are thought to be the result of decreased hepatic VLDL production and secretion[440] and increased post-prandial clearance.[340] Evidence from animal models can help explain why, in some cases, fish oil may increase LDL. Huff and Telford (1989) investigated effects of fish oils on lipoprotein turnover in pigs and reported increased production of smaller VLDL particles which are more likely to be converted to LDL.[441]

Controlled metabolic studies have found that fish oils prevent increases in plasma TG. The effect was largely attributed to inhibitory effects of fish oil on VLDL production. Reductions in a range of atherogenic lipoprotein particles including CM remnants, LDL, Apo B, C and E have also been reported with fish oil supplementation.[340, 403] For example DHA supplementation inhibits Apo CIII an inhibitor of lipoprotein lipase (LPL). LPL controls TG plasma clearance. Therefore restriction of Apo CIII by DHA enhances TG clearance.[442]

3.7.4.8 Summary of n-3 effects on the lipid profile

Dietary *n-3* fatty acids from fish and fish oil have profound hypolipidemic effects in healthy subjects and in HTG patients. Reductions in TG and VLDL are reported in association with EPA and DHA both combined and in separate formulations.

There is no evidence for the superiority of EPA or DHA when given singularly. LDL concentration is seen to increase in many studies and changes in particle size have also been reported.

Effects on HDL are less conclusive, in some studies HDL is seen to increase whereas others report no effects or decreases. The mechanism of the hypolipidemic action of the *n-3* LC-PUFA in fish oil is well documented.[440, 443] Synthesis of TG and VLDL in the liver is greatly reduced by *n-3* LC-PUFA as is the turnover time of VLDL in plasma.

In general *n-3* LC-PUFA reduce serum TG concentrations by 25-50%. Total cholesterol is not affected, LDL concentrations tend to rise by 5-10% but the more buoyant LDL sub-fractions associated with a less atherogenic phenotype may be favoured.[419] HDL cholesterol increases by between 1 and 17% improving the total to HDL cholesterol ratio. Effects on LDL particle size can be largely attributed to DHA rather than EPA.[299] Triglycerides and HDL cholesterol are widely accepted to be major determinants of LDL particle size.[299] However, TG is reduced by both EPA and DHA and it is therefore unlikely that TG is the major determinant of LDL particle size in the supplementation trials reviewed here. Rather, effects of *n-3* supplementation on proteins, with central roles in lipoprotein metabolism, for example cholesterol-ester transfer protein (CETP) and hepatic lipase, should also be considered when forming dietary guidelines.

It is possible that placebo oils used as comparators in many trials reported here may have produced independent effects on the lipid profile. For example, olive oil contains a high concentration of omega-9 MUFA which has been shown to have beneficial effects on CVD risk.[167, 444-447] Furthermore, *n*-6 PUFA is consistently reported to lower total and LDL cholesterol and in some studies HDL.[141] In view of this guidelines for fat intake have advocated MUFA in preference to n-6 PUFA as a replacement for SFA. The rationale for this is that *n*-6 PUFA may reduce both detrimental LDL and beneficial HDL. However, recent meta-anlayses have concluded that overall replacing SFA with *n*-6 PUFA increases HDL and decreases LDL whilst replacement with MUFA has no benefits for LDL. Replacement of carbohydrate or SFA with unsaturated fatty

acids produces a rise in HDL and this is greatest in association with MUFA.[447, 448] With respect to CVD, the most critical actions of *n-3* fatty acids are those that affect the vascular endothelium **(Chapter 2)**.

3.8 Summary and Conclusions

There is no doubt that dietary *n-3* fatty acids are important for health. Firstly, there is a dietary requirement for the parent *n-3* fatty acid, ALA, which is essential to health and cannot be synthesised within the human body. Secondly, longer chain fatty acids have important biological roles and although they can be produced from the parent *n-3* fatty acid ALA conversion is slow. Therefore there may be benefits from direct consumption of *n-3* LC-PUFA.

A large body of observational and experimental evidence supports the hypothesis that *n-3* fatty acids are important in primary prevention of CVD. However, evidence from RCTs is lacking and causality cannot be assumed. Data from controlled trials are equivocal and it is unclear whether supplemental *n-3* fatty acids influence CVD development either in people at high risk of CVD or in healthy populations. Data informing secondary prevention are more convincing and several large trials provide compelling evidence for a role of *n-3* LC-PUFA in protection from recurrent myocardial infarctions and death from CHD.[132, 196]

Dietary assessment has been poorly conducted in both primary and secondary prevention studies and few have used validated methods to assess *n-3* fatty acid intake. Therefore the role of dietary *n-3* in CVD risk is unclear. There is an urgent need for further well-designed randomised controlled trials investigating separate effects of LC-PUFA on primary CVD risk in young, healthy populations, unlikely to be affected by CVD. Such trials should incorporate rigorous and pertinent dietary assessment measures.

Clearly, more data are needed to inform public health advice targeted at primary prevention of CVD and clinical guidelines for secondary prevention. Data should ideally come from well designed RCTs. The aim of this present study was to conduct such a trial in which effects of DHA on vascular structure and function

were investigated in a group of young healthy adults, free from CVD and with minimal modifiable risk factors.

Interventions could include supplementation with *n-3* fatty acids or advice to increase dietary intake. In either case, accurate dietary assessment is essential to inform dietary strategies. A valid method for assessment of *n-3* fatty acid intake is needed and could prove a useful tool both clinically and in research. A further aim of this study was to develop such a tool.

Table 3-1 Controlled trials of EPA and DHA on the lipid profile

Author, setting, year	Study design	Mean Change from Baseline (%)					
		Diet/Dose (g/d)	Duration (weeks)	TG	LDL	HDL	VLDL
EPA & DHA							
Eritsland, Sweden, (1989)[414]	Randomised, single-blind, parallel study among patients with stable CHD (*n* = 22)	EPA/DHA (3.4)	4	-17	NR	NR	NR
Kestin, Australia, (1990)[418]	Randomised, single-blind, parallel study among normotensive men with mild HC (*n* = 33).	EPA/ DHA (3.4)	6	-39**	+9**	None	-58**
Simons, Australia, (1990)[415]	Randomised double-blind cross over study among patients with: Primary (*n* = 9) or Marked HT (*n* = 4).	EPA/DHA (2.8) (4)	12	-50	+18*	None	NR
Bonaa, Norway, (1992)[449]	Randomized, single-blind, parallel study among 156 healthy men and women, aged 34-60 years.	EPA/ DHA (6)	10	-21**	None	None	NR
Lungershausen Australia, (1994)[391]	Randomized double-blind placebo-controlled cross-over trial in hypertensive patients (*n* = 43).	EPA/DHA (3.4)	6	-21**	None	+15**	NR
Grundt, Norway, (1995)[450]	Randomised, double-blind, parallel study among patients with combined hyperlipidaemia (*n* = 57).	EPA/DHA (4)	12	-28*	None	None	NR
McKeone, Norway, (1997)[451]	Randomised, double-blind, parallel study among male and female patients with severe HT (*n* = 19).	EPA/DHA (4)	6	-26†	None	None	NR

Author, setting, year	Study design	Mean Change from Baseline (%)					
		Diet/Dose (g/d)	Duration (weeks)	TG	LDL	HDL	VLDL
Harris US, (1990)[438]	Prospective study of patients with HT (*n* = 10).	EPA/DHA (4.5) (7.5) (12)	18	-46** -54** -61**	None +23** +28**	None None None	-59** -65** -65**
Harris, US, (1993)[452]	Double-blind crossover design study of eight healthy volunteers.	EPA/DHA (1g/10kg BWT)	4	-40**	<1	<1	NR
Harris, US, (1997)[439]	Prospective, double-blind, placebo-controlled trial among patients with HT (*n* = 42).	EPA/DHA (4)	16	-45	+31	+13	NR
Sirtori, Italy, (1998)[421]	Prospective, double-blind, placebo-controlled trial among patients with HT and other CV risk factors (*n* = 935).	EPA/DHA (2)	24	-21**	None	+8	NR
Stalenhof, Netherlands, (2000)[417]	Randomised, double-blind, parallel study among patients with HT (*n* = 28).	EPA/DHA (4)	12	-37**	+29**	+11**	-49**
Calabresi, Italy, (2000)[419]	Randomised, double-blind, crossover study among patients with HC (*n* = 14).	EPA/DHA (3.4)	8	-27†	+21†	+15†	-18
EPA only							
Grimsgaard Norway, (1997)[425]	Randomised, double-blind, parallel study among healthy men (*n* = 234).	EPA-EE (4)	7	21**	None	None	NR

Author, setting, year	Study design	Mean Change from Baseline (%)					
		Diet/Dose (g/d)	Duration (weeks)	TG	LDL	HDL	VLDL
[1]Mori, Norway,(2000)[429]	Randomised, double-blind, parallel study among overweight and dyslipidaemics (59).	EPA-EE (4)	6	18	None	None	NR
Woodman, Australia, (2002)[427]	Randomised, double-blind, parallel study among hypertensivesand type 2 diabetics (*n* = 59).	EPA-EE (4)	6	-19*	None	None	NR
Nestel Switzerland, (2002)[428]	Randomised, double-blind, parallel study among patients with dyslipidaemia (*n* = 41)	EPA (3)	7	-23*	None	+5	-29**
Yamamoto, Japan, (1995)[404]	Randomised, non-blind, parallel among healthy individuals (*n* = 22, 12 intervention v 12 control).	EPA (1.8)	16	-6	+2	+3	NR
DHA only							
Conquer , Canada, (1996)[407]	Randomised, double-blind, parallel study among healthy vegetarians (*n* = 24)	DHA (1.62)	6	-9	-7*	+17**	NR
Conquer, Canada,(1998)[406]	Randomised, double-blind, parallel study among healthy subjects of Asian Indian background (*n* = 22, 14 male).	DHA (0.75) (1.5)	6	-17 -6	-9 None	+5 +5	NR NR
Hamazaki, Japan, (1996)[410]	Randomised, double-blind, parallel study among healthy vegetarians (*n* = 24).	DHA FO (1.5-1.8)[a]	24	None	None	None	NR
Agren, Netherlands, (1996)[409]	Randomised, single-blind study among healthy males(*n* = 55).	DHA FO (1.68)	15	-15	+9	0	NR

Author, setting, year	Study design	Mean Change from Baseline (%)					
		Diet/Dose (g/d)	Duration (weeks)	TG	LDL	HDL	VLDL
Grimsgaard Norway,(1997)[425]	Randomised, double-blind, parallel study among healthy men (*n* = 234).	DHA-EE (4)	7	26**	None	+4***	NR
Nelson,US, (1997)[431]	Randomised, single-blind trial among healthy volunteers (*n* = 6).	DHA (6)	13	-12**	-1	-9	NR
Mori, Australia, (2000)[453]	Randomised, double-blind, parallel study among overweight male patients with dyslipidaemia (*n* = 59).	DHA 4g	6	20**	+8	None	NR
Woodman, Australia, (2002)[432]	Randomised, double-blind, parallel study among hypertensives and type 2 Diabetics (*n* = 59, 39 male).	DHA-EE (4)	6	-15*	None	12*	NR
Nestel, Switzerland, (2002)[428]	Randomised, double-blind, parallel study among dyslipidaemics (*n* = 38).	DHA-EE (3)	7	-32	+5	+9	31**
Geppert , Germany, (2006)[432]	Randomised, double-blind, parallel study among healthy vegetarians (*n* = 106, 27 males).	Algal DHA (0.94)	8	-23***	+11***	+7**	NR
Wu, Taiwan, (2006)[433]	Randomised, single-blind, parallel study among healthy female vegetarians (*n* = 27).	Algal DHA (2.14)	6	-18	-3	+6	NR
Sanders, UK, (2006)[411]	Randomised, double-blind, parallel study among healthy Vegetarians (*n* = 79).	Algal DHA (1.5)	4	-14*	+7*	+10*	NR

Author, setting, year	Study design	Mean Change from Baseline (%)					
		Diet/Dose (g/d)	Duration (weeks)	TG	LDL	HDL	VLDL
Davidson, US, (1997)[412]	Randomised, double-blind, parallel study among hyperlipidaemics (*n* = 27).	TG-DHA (1.25) (2.50)	6	-21 -18†	+9 +14	+6	+6
Engler, US, (2004)[434]	Randomised, double-blind, crossover study among children with familial hyperlipidaemia (*n* = 20).	DHA (1.2)	6	-5	+8	+5	
Kelley, US, (2008)[413]	Randomised, double-blind, parallel study among male patients with hypertriglyderidaemia. (*n* = 34).	DHA (3.0)	12	-24**	+15**	+7**	NR

Abbreviations: HT, hypertriglyceridaemia; HC, hypercholesterolemia; EE, Ethyl Ester; FO, fish oil.* Statistically significant difference from baseline values ($P \leq 0.05$) or ** ($P \leq 0.01$) or *** ($P \leq 0.001$), †=significance not reported, [1] =Comparison with placebo, NR=not reported.

Chapter 4

Dietary Assessment

How much cheese *is a handful? How much more or less is a cupful? What is the capacity of a glass, a tumbler, or a soup ladle? What is the difference between a suspicion and a pinch? How much more is a good pinch? How* much wine *is a little, how many olives a few? When a book says a tin of chopped almonds or pomegranate juice what are you supposed to understand by that?"*
Elizabeth David (1913-1992)

4.1 Introduction

The importance of nutrition for health was first recognised through associations between diet and deficiency diseases such as scurvy, beriberi and rickets and stunting in children. The observation of the famous British naval surgeon, James Lind (1716-1731), who noted in a group of sailors attending the HMS Salisbury, that "they all in general had putrid gums, the spots and lassitude, with weakness of their knees" amounts to the first description of scurvy, a disease resulting from deficiency of ascorbic acid more commonly known as vitamin C.[454] Similar observations among sailors led to the identification of further diseases arising from nutritional deficiencies. For example, sailors living on a diet based on refined rice developed signs of a disease termed beriberi which was later attributed to thiamine deficiency.[455]

Pellagra, which results from deficiency of niacin, was first recognized in early settlers of the US who existed on a restrictive diet based largely on corn meal.[455] Their failure to use traditional food preparation methods, established by native cultivators, led to the trapping of niacin within corn kernels which reduced its bioavilability and led to deficiency. Although unable to describe the mechanism for this, Native Americans knew that treating corn with lime, an alkaline solution, prevented disease, enabling them to remain healthy on a predominantly corn meal diet. Early priorities in healthcare therefore focused on defining and providing nutritionally-adequate diets that could support growth and development and maintain health.

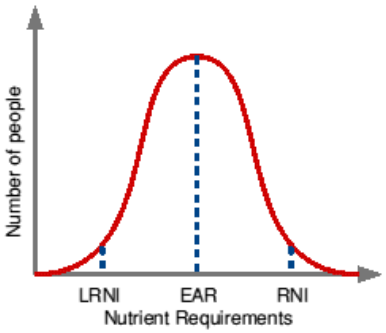
Defining nutritional intakes for groups of people within specific populations, however, is a challenge for public health policy makers. In 1987 the UK Committee on Medical Aspects of Food and Nutrition Policy (COMA) reviewed its Recommended Daily Amounts (RDAs) of food energy and nutrients for people in the UK. The panel moved from RDAs to Dietary Reference Values (DRVs) with the aim of giving clearer guidance (DH, 1991).[159]

4.2 Dietary Reference Values

Assessment of dietary adequacy is usually made by comparison with a reference that defines a level of intake required to prevent deficiency. For example, in the UK, guidelines for the general public reflect recommendations for nutrient intake based on advice from COMA and the Scientific Advisory Committee on Nutrition (SACN). These recommendations are based on conclusive evidence where possible.

In 1991, the Department of Health published DRVs which cover a range of intakes for most nutrients.[159] Dietary recommendations are usually set at a level considered adequate for almost all healthy people within a population, and for many nutrients the ideal recommended intake termed the reference nutrient intake (RNI) is set at two standard deviations above the estimated average requirement (EAR). The RNI is considered adequate for most (97.5%) of the population. The EAR represents an amount that will meet the needs of 50% of the population. A lower reference nutrient intake (LRNI) is set at two standard deviations below the EAR and would meet the needs of only a very small percentage of the population (2.5%) with the lowest nutritional needs. This model assumes that individual requirements are normally distributed **(Figure 4-1**).

Figure 4-1 Graphical representation of dietary reference values (dtonline.org)



4.3 Nutritional Epidemiology

Understanding diet-disease relationships is crucial in the identification and management of nutrition related disease that can arise from both nutritional deficiency and excess. Studies of diet and disease can provide evidence to inform public health. However, it is essential that reliable dietary assessments are made in such studies as public health recommendations should only be based on reports that include valid measures of nutritional intake i.e. that have an acceptable level of accuracy and precision.[456] This is also important in clinical practice where accurate dietary assessment is needed to inform appropriate nutritional interventions to redress deficiencies or excesses.[457] Most nutrition research studies are conducted within populations – termed nutritional epidemiology.

Nutritional epidemiology evolved out of the necessity to understand the role of diet in disease among populations. The science has a long history and evidence for the role of diet in the development of chronic disease first emerged in the scientific literature as far back as the eighteenth century.[458] Large scale studies of food consumption began in the 20th century with dietary surveys. These were designed to provide information about what people ate and aimed to provide a basis for improving dietary practices. Dietary surveys are complex and there are many practical problems in their planning, organization and implementation. For example, reconnaissance of the survey area is needed to glean information about the environment and socio-demographic setting. Statistical planning is essential

to the selection of an appropriate representative population sample and, perhaps most importantly, foods must be described accurately and the calorific and nutrient values of as wide a range of foods as possible calculated and compiled into food composition tables. The importance of comprehensive nutrient databases and their limitations are discussed in **Section 4.5.3.4**. The design of studies requiring dietary assessment varies depending on their study objective. Possible study designs used in nutrition research studies are discussed below.

4.3.1 Descriptive Epidemiological Research

Observational studies allow the investigation of relationships between dietary exposures and disease outcomes. Possible designs include cross-sectional, cohort or case-control studies.

4.3.1.1 Cross-sectional Studies

Nutritional surveys such as the National Diet and Nutrition Survey (NDNS), previously carried out periodically (approximately every 10 years and now a rolling programme) in the UK, are usually cross sectional. Data collected in such surveys inform associations between food and/or nutrients or dietary patterns and health outcomes both within and between populations. Dietary assessment methods used in national surveys are mainly quantitative and allow estimations of both type and quantity of foods (including beverages) and nutrients consumed at the population level. For example, in the UK NDNS, seven days of weighed records are collected.[160] Multiple day weighed food records are considered to be the "gold standard" method that is closest to the truth and likely to provide the most valid estimate of dietary intake.[459] However, in reality there is no gold standard for measurement of dietary intake as all methods are prone to bias.[460]

4.3.1.2 Correlation Studies

As well as population surveys, food consumption studies investigate relationships of diet with specific disease outcomes. Throughout the last and present century many large scale studies have been instigated with this specific aim. Early epidemiological investigations of diet and disease are mainly correlation studies that compare disease prevalence with consumption of specific foods. Examples

include the work of Armstrong and Doll (1975) that described the relationship between meat consumption and the incidence of colon cancer among women from 23 countries.[461]

Interpretation of correlation studies is hampered by the existence of confounders affecting disease risk that may also vary between areas of high and low incidence. Furthermore, most surveys used food disappearance data, which make assumptions about food consumed by individuals on the basis of what is bought. Surveys do not always take into account wastage or extra foods consumed from household stores. Therefore, as all foods bought may not necessarily be consumed by the study population assessments based on household food shopping records do not accurately reflect intake.

4.3.1.3 Case-control Studies

An alternative approach to cohort studies is the case-control study design in which subjects are recruited on the basis of the presence or absence of outcomes of interest. This enables comparisons between groups of individuals with differing dietary habits. Many studies investigating diet-disease relationships use retrospective methods to assess dietary intake in individuals developing specific disease states. Such studies are strongly biased by participants' abilities to accurately recall past dietary intake.

In some studies exposure is determined prospectively and is therefore free from recall bias. However, other sources of bias are likely in these studies including behavioural changes that may occur as a result of being observed **(Section 4.5.3)**.

4.3.1.4 Limitations of Descriptive Epidemiological Studies

The complexity of diet further hampers the study of diet-disease relationships. Diet is not a single exposure but rather a collective of multiple exposures. For example, within a population individuals eat many of the same foods which in turn contain the same nutrients. Therefore, many or all individuals within the study population may be exposed to the same hypothesized causal agents. Dietary

behaviours also tend to cluster in sub-groups that share similar lifestyle characteristics such as income, smoking and alcohol habits. Ethnicity and familial risk factors are additional correlates with dietary behaviours and these factors may collectively or independently influence the risk of developing chronic disease. It is important to control for possible confounding variables in analyses of diet disease relationships. However, the aim of epidemiological studies is to study heterogeneous populations where possible to gain the most accurate assessments of diet in free living individuals.

Effects of nutrition on disease usually develop over time. Therefore the time lag between previous dietary exposures and disease development may occlude causal relationships. Cross sectional and retrospective cohort studies are therefore limited in their ability to detect diet-disease relationships. Observational studies are further complicated by possible changes in dietary habits that may occur during the period between dietary exposure and disease development. For example, a lack of correlation between dietary fat and serum cholesterol concentrations was reported in studies from the United States.[462] Conversely, cohort studies have consistently reported relationships between dietary fat and specific fatty acids with increased risk of CVD.[463]

One major disadvantage of observational epidemiological studies is that they are unable to determine causality; an experimental study design is needed for this. However, it is not always ethical to test hypotheses about diet/disease relationships. For example, a wealth of epidemiological data supports the hypothesis that diets high in saturated fat increase the risk of CHD and it would therefore not be ethical to randomize human subjects to high fat diets to make comparisons with respect to health outcomes. In this instance prospective cohort studies are more likely to provide useful data on habitual diet that can be correlated with health outcomes.

Prospective cohort studies are useful in generating hypotheses that can inform experimental research. Once identified, a diet or dietary pattern that is strongly associated with health benefits can be applied to experimental research. For example, it is possible to compare diets with recognized health benefits, such as

a Mediterranean style diet with a diet common to a population. Limitations of such studies include the likelihood that effects on disease development attributable to diet may be small and studies investigating diet-disease relationships may therefore require large sample sizes. Furthermore, adherence to specific diets by healthy free-living individuals is difficult to achieve.[241]

4.3.1.5 Experimental Nutrition Research

Experimental research allows specific hypotheses about diet-disease relationships to be tested and the most robust study design is the RCT which ideally should be conducted as a double-blind experiment. In this type of study effects of potentially confounding variables are minimized through random allocation of participants to intervention or placebo groups. With this study design it is possible to create differences between groups with a magnitude sufficient to make comparisons of effects attributable to a specific exposure. Following intervention, groups are compared and differences between outcomes of interest assessed. This allows estimation of disease risk in relation to exposures (e.g. nutrient or food) of interest.

Experimental studies among humans are expensive to conduct and should only be considered after evidence from animal models or from observational epidemiological human studies has generated hypotheses that warrant further investigation. RCTs lend themselves most appropriately to investigations using single nutrients that can be provided as pills or capsules.[464] However, interventions incorporating advice to follow specific dietary guidelines have also been widely used.[465]

Whatever study design is selected, in all nutrition research assessment of diet is essential. However, collecting accurate information about dietary intake is notoriously difficult to achieve.[466] No dietary assessment method has yet been identified that is completely accurate, precise, affordable and feasible for use in both experimental and observational studies. The challenge to researchers is to collect information about dietary intakes that are as close to the truth as is reasonably possible within the constraints of the study. The aim of the present study is to investigate the relationship of diet to the risk of developing of CVD

which requires knowledge of food consumption at the individual level. The following sections therefore focus on dietary assessment methods used at an individual level.

4.4 Dietary Assessment Methods

To examine the relationships between diet and disease, dietary assessment methods are needed that can identify individuals consuming specific foods and nutrients of interest or who can be characterised by specific dietary patterns. It is essential to clarify the study aims and thus identify the type of dietary information required. Methods can be either quantitative or qualitative as dictated by the research question. This thesis used both quantitative and qualitative methods to investigate relationships between diet and vascular function.

4.4.1 Quantitative Dietary Assessment

Various methods of quantitative dietary analysis can be used depending on whether the intention is to: 1) describe the mean intake within the study population; 2) determine the proportion of a population at increased risk; 3) categorise an individual on the basis of their level of intake; or 4) describe absolute intake in individuals. Methods 1 and 2 are useful for reporting intake at the group level; for example to evaluate the level of intake or identify the proportion of the population at risk of deficiency following supplementation with foods or nutrients or fortification of specific foods. Methods 3 and 4 are more useful in a clinical setting, to identify individuals' levels of disease risk. For example, in this thesis classification into divisions of population intake is used to identify low and high consumers of *n-3* LC-PUFA and examine relationships with CVD risk.

The quantitative approach to dietary assessment requires methods that can correctly classify individuals according to their level of nutrient or food intake with an acceptable degree of accuracy. This is feasible provided the dietary assessment method's relative validity has been assessed against a superior measure of intake. Describing the absolute magnitude of intake for a specific

food or nutrient is the most error prone assessment to attempt and therefore qualitative dietary assessment has gained popularity in recent years.

4.4.2 Qualitative Dietary Assessment

There is a growing consensus that a more holistic multifaceted approach is needed to better understand the relationship between diet and health particularly for assessment of dietary behaviours in free living humans.[467] In reality, people eat meals made up of a combination of foods, which are themselves a complex mixture of nutrients. Furthermore, foods are eaten in different combinations and in different ways. For example, meal pattern varies greatly with some preferring traditional patterns, based on 3-4 meals per day and others inclined to 'graze' on more frequent eating episodes.

4.4.2.1 Dietary Pattern Analysis

Recently, dietary pattern analysis has emerged as an alternative or complementary approach to examine the relationship between diet and the risk of chronic diseases. Instead of looking at individual nutrients or foods, dietary pattern analysis examines the effects of overall diet. Dietary patterns represent a broader picture of food and nutrient consumption, and may thus be more predictive of disease risk than individual foods or nutrients.

Two methods are most frequently used to derive dietary patterns. One approach is to use scores or indexes based on *a priori* hypotheses about the role of dietary factors in disease prevention.[468] One pattern that is universally accepted as beneficial in CVD prevention is the Mediterranean diet. The degree of adherence to Mediterranean style dietary patterns can be estimated using scoring systems of which the most widely used is the Mediterranean diet score. The original Mediterranean diet score proposed by Trichopoulo *et al.,* (1995) comprised eight components: high MUFA to SFA ratio; high consumption of legumes; cereals (including bread and potatoes); fruits and vegetables; low consumption of meat and meat products; and milk and dairy products, and moderate alcohol consumption.[469] Subsequent scores have been modified to accommodate local dietary habits and secular changes (e.g. tMED,[470] aMED,[471] or rMED,[472]). In

recent meta-analyses of studies using such scoring systems, a higher degree of adherence to the Mediterranean diet was associated with a reduction in CVD mortality.[186, 187] Effect sizes ranged from a relative risk of 0.4 to 0.9.

A second approach is to derive dietary patterns from dietary assessment data using principal component analysis (PCA). The aim of this technique is to reduce the number of foods in a dataset by grouping foods of similar characteristics together. PCA attributes a score or factor loading to food groups based on the sum of the daily frequency of intake of foods comprising each group. This identifies patterns characterised by significant consumption of specific foods. A detailed description of PCA is given in **Chapter 6, Section 6.5.9**. Dietary patterns derived from these methods have been examined in relation to nutritional and disease biomarkers and various health outcomes, and generally show the desirable dietary pattern to be consistent with prevalent beliefs about what constitutes a "healthful" diet. Observational studies suggest that "healthful" dietary patterns are associated with significant but modest risk reduction (15%-30%) for all-cause mortality and CHD.[473]

More recently a third method in dietary pattern research has emerged. Ranked Regression Analysis (RR) uses data from two types of observed variables known to be related to outcomes of interest to derive dietary patterns. In RR food group data derived from FFQ can be used in conjunction with biomarkers of disease status. For example Liese and colleagues (2009) used RR to examine relationships between dietary patterns and common carotid artery IMT.[281] A set of dietary variables were derived from FFQ and combined with a data set describing fibrinogen and plasminogen activator inhibitior 1 concentrations, recognised CVD risk factors, in the same subjects. The mathematical basis of RR is similar to factor analysis but it is applied to two sets of variables as opposed to only one.[281]

Defining a "healthful" or "healthy" diet in specific terms is a challenge for health professionals and researchers. Most countries have produced national dietary guidelines that offer general advice regarding foods that constitute a healthy diet and the proportions in which they should be eaten. For example in the U.K. the

eatwell plate is a policy tool that is used to depict the Government's recommendations on healthy diets **(Figure. 4.2)**. The model aims to make healthy eating easier to understand by giving a visual representation of the types and proportions of foods needed for a healthy and well balanced diet. Dietary messages are notoriously difficult to convey and are interpreted and acted on differently by individuals. Furthermore, compliance with dietary recommendations is shown to be poor in national diet and nutrition surveys.[160] Therefore, variance in day-to-day nutritional intake is likely to be high among individuals.

Figure 4-2 The Eatwell Plate (used with permission from the UK Food Standards Agency)

Even though much progress has been made as a result of the development of ever more sophisticated instruments and methods for dietary intake assessment, the relationship between diet and health is still not fully understood and no perfect dietary assessment method exits. Methods that aim to provide fully or semi-quantitative dietary intake assessments are discussed below.

4.5 Dietary Assessment Tools

There are a number of methods available for dietary assessment. These fall largely into two categories: prospective and retrospective There are several dietary assessment tools available for assessing nutrient intakes; these are summarised in **Table 4-1** and briefly described below.

4.5.1 Prospective Methods of Dietary Assessment

Prospective dietary assessment methods collect data recorded at the time of eating.

4.5.1.1 Weighed Records

The weighed records method is generally considered to be the most accurate prospective method of dietary assessment.[474] However, it is not practical for use in many studies as it is expensive, time consuming and places a considerable burden on both study participants and investigators. For example, Bingham and colleagues (1997) reported that 16 days of weighed records were necessary to assess usual dietary intake.[459] Moreover, despite their rigour, weighed records often highlight underreported energy and nutrient intakes due to participants neglecting to collect intake data comprehensively.[475, 476] Secular changes that include an increase in the amount of food consumed outside the home make this method increasingly more difficult. The weighed record method requires participants to provide detailed description of foods and beverages consumed on a meal by meal basis. All foods and beverages intended for consumption must be accurately measured against an appropriate scale. Amounts consumed can then be calculated by deducting food or drink left over and recorded in a booklet provided by the researcher. The main disadvantage of the weighed record method is that it places a considerable burden on participants who must be both literate and numerate.[477] Although methods have improved greatly in recent years, for example, with the provision of digital 'smart' scales able to automatically record and log food items, weighed intakes still place considerable burden on study participants. Moreover, the practice of detailed food recording is likely to introduce bias. For example, participants may change their eating habits to make the process less time consuming, or simply to appear compliant.

4.5.1.2 Observed Weighed Inventory

The observed weighed inventory method is the most arduous method for the researcher. All foods and beverages offered must be weighed before the meal is taken and all non-consumed food weighed at the end of the meal. The advantage is a high level of accuracy in quantifying amounts consumed.

Disadvantages include a high risk of bias as the participants are observed and therefore likely to change usual behaviours or to be influenced by the presence of the researcher. The risk of bias in nutrition research studies is discussed more fully in **Section 4.5.3**.

4.5.1.3 Estimated Food Records

Estimated food records rely on participants' reports of amounts of food and drink consumed based on comparisons with household measures. The amount of food eaten is described in spoons or cups, measured with a simple ruler or compared to a recognised household object. For example a piece of cheese may be compared to a matchbox, a potato to a standard egg etc. Food models, scales and photographs can all be useful in improving dietary assessments **(Figure 4-3)**. Photographs of average portion sizes tend to result in underestimates of portion size. However, Nelson and colleagues reported that the use of a photographic atlas illustrating a range of portion sizes was associated with relatively small errors (Nelson, et al., 1994).[478] Clearly, however, assessments can only provide an approximation of dietary intake.

Figure 4-3: Tools used to improve dietary intake estimates (author's copyright)

4.5.2. Retrospective Methods of Dietary Assessment

Retrospective dietary assessment methods collect data about food eaten in the (usually recent) past.

4.5.2.1 Twenty-Four Hour Recall

An alternative method which may be more feasible in research studies, is the 24-hour recall method. This involves the collection of single or multiple records of dietary intake based on the previous day's intake. Pioneered by Burke (1947),[479] this widely used method shifts the burden away from participants and towards the investigator who is able to collect comprehensive information about dietary intake over the 24 hour period preceding interview. Careful questioning allows investigators to make estimates about portion size and help quantify intake.

Information can be collected at personal interviews or by telephone. Twenty-four hour recalls conducted by telephone have been described by several investigators.[480-482] Morgan and colleagues (1987) compared the use of telephone with personal interview dietary assessments and concluded that the telephone interview provided comparable data with less effort and cost. Response rates were 71-81% for telephone interviews compared with 72–83% for personal interviews.[483]

A major advantage of this method is that it can be completed in a short time (10-20 minutes) and places minimal burden on study participants. However, this will vary according to participants' abilities and lifestyles. For example a less literate person may take longer to describe their dietary intake and an individual who prepares food from scratch may also need longer to describe ingredients, cooking methods etc. Previous researchers have reported a range of 5-67 minutes as necessary for 24-hr recall collection.[484]

As with all dietary assessment methods there are certain limitations. For example, a single 24-hour recall will provide a "snap shot" reflecting only a single day's intake and is unable to offer a valid estimate of habitual intake.[485] This can be overcome by collecting several days of records that will capture information on

day to day dietary variation and aid description of habitual diet.[486] Representation of all days, including weekend days is important as variability in food intake has been reported to be higher on weekend- compared with week-days.[487] Although weekend collections may increase investigator workload it is likely that more foods habitually eaten will be captured.[488, 489] Where possible, attempts to include assessments made during different seasons should be made. Seasonality affects food availability in some populations and intake differs according to the time of year for various reasons.

Where the aim is to collect multiple 24-hour recall records this is best done by the same researcher to reduce inter-operator variability. Although variability between investigators can be considerable, standardisation of methodology, for example the use of training protocols for interviewers, has been shown to reduce inter-operator variation. Detailed examples of training protocols used in the US National Heart Lung and Blood Institute studies that included extensive dietary assessment can be found online.[490]

The 24-hour recall method has been successfully used in many studies including national dietary surveys[491 492 493] and large intervention trials such as MRFIT.[494] It has been validated in several populations including elderly housebound adults,[495] British school children[496] and overweight adult men in the US.[497] In summary, the 24-hour recall method can be relatively inexpensive, places a lower burden on study participants compared with weighed intakes and may be feasible for large scale studies.

4.5.2.2 Dietary History

The dietary history method involves a face to face interview conducted by the investigator who is usually a dietitian or nutritionist. This method is widely used in clinical practice to help dietitians make assessments about nutritional adequacy. During the interview, the investigator uses an open question technique to gain insight into usual dietary practises. Examples of questions include: *"When do you usually have your first food or drink Mrs Brown?"* Using this method, the investigator attempts to avoid leading the interviewee into providing answers they may want to hear such as, for example, *"I always eat breakfast and have a*

healthy bowl of wholegrain cereal." An advantage of dietary history over dietary recall is that the interviewer collects information relating to habitual intake rather than a record of a given day's intake. This may be more useful in studies where the aim is to assess diet disease relationships. However, where assessment of temporal relationships between nutrient intake and outcomes is required a dietary recall may be more appropriate. The diet history method also allows detailed questioning to collect information about portion size, cooking methods etc. Disadvantages include a high level of dietary expertise from the interviewer and a considerable time commitment from the participant which therefore make this a costly option.

4.5.2.3 Food Frequency Questionnaire

Semi-quantitative information can be collected using dietary questionnaires. Food frequency questionnaires (FFQ) are used to provide retrospective information about which foods or food groups are regularly eaten and the frequency of their consumption. This information can then be used to provide estimates of food and nutrient consumption or to describe dietary patterns.

FFQs require participants to indicate their frequency of consumption of certain foods. The questionnaire can be either self- or investigator-administered. In either case, a detailed explanation is required before completion to ensure accurate reporting. FFQs can be sent out to participants with detailed instructions. Ideally, this should be supported by verbal instructions given by telephone. Preferably, FFQs can be administered while participants attend the research centre. In this scenario investigators can provide more detailed explanation and instruction and provide help during the completion process.[498]

Advantages of FFQs include: 1) they are usually self-administered so place less burden on researchers; 2) they take less time to complete than detailed food records; 3) less time is needed for coding and entry to nutrient analysis programmes. They may also be more appropriate in some study designs, for example when information about habitual diet or dietary patterns is required.[499] Disadvantages include: 1) FFQs are semi-quantitative only; 2) they tend to underestimate energy and macronutrient intake[500] and overestimate micronutrient

intake[501] and 3) there is loss of detailed information as foods are grouped together.

In general, FFQs are widely accepted to be the most appropriate method for dietary assessment in studies of diet-disease relationships.[458] However, it is important to consider whether the FFQ is appropriate for use in the population under study. For example has the FFQ been validated in the study population or a comparable sample. During the 1950s investigators developed FFQs and evaluated their role in dietary assessment.[502-503, 474] Heady (1961) was the first to demonstrate that reported frequencies of food intakes was highly correlated with weights consumed over several days.[504] The earliest practical application of the FFQ method was in 1976 when Nichols and colleagues used an FFQ in the Tecumseh Heart Study.[505] In this study, failure of the FFQ to detect an association of fat intake with serum cholesterol led to decreased interest in the use of FFQs. However, since this study, serum cholesterol has been identified as a poor indicator of dietary change because it does not accurately reflect short term dietary fat consumption.[107] Therefore, the biochemical marker, rather than the dietary assessment tool may have been the limiting factor.

In summary, weighed records have been shown to be the most accurate quantitative method of dietary assessment. However, they are expensive, time consuming, invasive and therefore impractical for use in many research studies and clinical assessments. A major quest of nutrition epidemiology has been to identify dietary assessment methods that are able to assess usual diet and yet be suitable for large numbers of participants. This requires demonstration of validity of other methods, traditionally against the "gold standard" weighed food record. Validation is particularly important in dietary assessment studies because methods are prone to error. These are discussed in the following section.

4.5.3 Sources of Error in Dietary Assessment

> *"Accurate estimates of the dietary intake of free-living individuals are required for nutritional research, epidemiology, and clinical medicine. However, all dietary assessment techniques rely on information supplied by the subjects themselves and so the validity of results is uncertain"*
>
> *(Bingham & Cummings, 1985).*[506]

Measurement errors in dietary assessment occur both randomly and systematically. Random errors may be introduced by day-to-day variations in dietary intake or as a result of measurement error. Whether fluctuation results from genuine day-to-day variations or measurement errors, provided an adequate number of daily records are collected per individual the average value from repeated measures will approximate the true value.[458] This is not true for systematic errors, however, which occur for example when dietary intake reporting is influenced by participation in a study, a phenomenon known as the Hawthorne effect.

The Hawthorne Effect was first reported following research investigating methods of increasing productivity in the Western Electrical Company's Hawthorne Works in Chicago during the 1920's and 30's. The finding of interest was that no matter what change was introduced to working conditions, the result was increased productivity.[507] When applied to nutrition research the Hawthorne effect may lead individuals to either over- or under-report intake of certain foods and distort the truth. Standardised questionnaires are particularly prone to systematic errors. For example, when a food regularly consumed by an individual is omitted or a question misunderstood the truth cannot be reached even with collection of multiple records.

Errors in dietary assessment affect both within and between subject measurements. There are three major sources of error in dietary assessment methodologies: 1) respondent; 2) interview and 3) database errors. These are discussed briefly below in **Section 4.5.3.1 – 3**.

4.5.3.1 Respondent errors

Researchers report a variety of influences on the ability of participants to provide accurate reports including, educational, social and motivational factors.[508] [509] Age, sex and environment have important influences on food intake memory. For example recall is reported to be better in women compared with men and in younger compared with older individuals.[510] To provide reliable details of dietary intake respondents must have a reasonable knowledge of food, good communication skills and a reliable memory. Memory aids can be helpful in

obtaining accurate information regarding foods and the quantities eaten. For example, prompts can be used to help the respondent's memory. With careful probing the interviewer can uncover snack foods eaten whilst watching television or socialising. Checklists are also useful and can help respondents feel secure in reporting foods perceived as treats. Furthermore, suggesting named foods can help recall. However, it is important to suggest at least 3 alternatives to minimise bias. Visual reminders can also be helpful and food models and packaging are often used as memory aids. These have the added advantage of assisting with portion size estimation.

With the exception of weighed records and many (non-quantitative) FFQs, participants must also be able to estimate quantities of foods and beverages consumed. Although descriptions of portion size may help achieve a more accurate assessment, this is frequently estimated inaccurately and a high degree of variation between perceived and actual standard portion size is commonly reported.[511, 512] Some researchers have reported photographs as useful aids to improve estimation of food portion size.[511, 513] Guthrie (1984) reported an error margin greater than 50% where no aids were used in assessments of food portion size.[511] Similarly, Pietinen and colleagues (1988a & 1988b) reported that the use of photographs improved agreement between semi-quantitative assessment methods and weighed records.[514, 515] More recently, Nelson and colleagues compared the use of photographs depicting average portion size with those depicting a range of portion sizes and found that the use of a series of eight photographs greatly reduced errors in size perception.[478, 516]

4.5.3.2 Interview errors

Errors that occur during the interview process arise for several reasons and may be attributed to the interviewer, the participant or a combination. One of the main sources of error in dietary assessment is misreporting, comprising both under- and over-reporting. Misreporting introduces severe error not only in the estimation of energy intake, but also in that of other nutrients, and may be a result of participants' poor ability to accurately recall intake and to describe portion size.[517] Memory is highly influential and may be suppressed or distorted

either of which may result in the unintentional omission or addition of foods.[518] Emphasis should be placed on careful questioning using open style interviews **(Section 4.5.2.2)**. One strategy to reduce error is to incorporate quality control assessments for interviewers participating in large scale studies. For example, in the International Collaborative Study of Macro- and Micronutrients and Blood Pressure (INTERMAP) study dietary interviewers were intensively trained and recalls taped. Recordings were used to highlight areas requiring retraining or work to improve performance.[519]

4.5.3.3 Coding and Database errors

After collection, dietary records must be coded and entered to a nutrient database. A codebook is essential to ensure standardisation. This should give food codes and advice as to which specific foods they should be used for. In some cases, decisions regarding which code to use will be straightforward whilst in others nutrition knowledge and training is essential in the selection of the most appropriate food code. For example in most cases a boiled egg is simply that and usually only one code will be appropriate but for other foods, that can be prepared or cooked in several ways, judgements must be applied. A comprehensive code book can help guide decisions for example by prompting data entry staff to check for additional information. Where this information is not available standard (default) coding decisions can be applied.

Where a quantified assessment is required food densities and average portion size information is also required. In some cases additional information may be needed to determine the most appropriate code. For example, details of usual food preparation and/or cooking methods can aid the food code decision.

There are several stages in the coding process where errors can be introduced. Firstly, food codes may be inappropriate. For example, if a code allocated to a particular food has not been analysed for the nutrient of interest, total intake for that nutrient will be underestimated. Secondly, mistakes may occur in the coder's interpretation of the food record. For example, the study participant reports daily consumption of a specific food but the coder omits to refer to additional information. This could lead, to the selection of a food higher in a certain nutrient

than that actually consumed. For example, additional information indicates that the participant removes excess fat from meat; in this case the coder should have the opportunity to select a food code that reflects this. However, failure to use additional information may lead to an incorrect choice of food code. Thirdly the coder misreads or wrongly transcribes information from the code list to the data entry sheet and the wrong food is entered to the dietary analysis programme.

For the purpose of epidemiological studies the food composition database should be comprehensive, complete and current. Information on all nutrients and other food constituents is essential and the database should be updated to reflect secular changes in food consumption. Dietary analysis programmes available in the UK are based on the data set of foods and nutrients in McCance and Widdowson's The Composition of Foods and its supplements published by the Royal Society of Chemistry (McCance and Widdowson's The Composition of Foods, 5th Edition, 1993.[520]

4.5.3.4 Sources of Error in Food Composition Analysis

The earliest analyses for McCance and Widdowson's Composition of Foods began early in the previous century when McCance first identified the need for accurate quantification of carbohydrate in the management of diabetes. Recognising that the currently available American food tables omitted to separate available from non-available carbohydrate, McCance embarked on the production of a more accurate food database, based on UK foods. With a grant from the UK Medical Research Council, he began with the analysis of fruits and vegetables (McCance in McCance and Widdowson's The Composition of Foods, 5th Edition, 1993).

A database designed for the purpose of food analysis should include raw and cooked foods and ideally branded samples and recipes. Sampling is an important consideration and the quality of data should ensure *'fitness for the purposes for which it should be used'*.[521] Values in McCance and Widdowson's were derived from direct analysis of foods using carefully designed sampling protocols. Most values were derived from pooled samples where foods purchased from different shops were combined. Ten samples were used in most

cases. These were typically chosen to account for variations within product ranges including ratio of baked beans to tomato sauce etc. Therefore, values in these food tables have been derived from careful analyses and are representative of foods commonly consumed in the UK. However, it is important to appreciate that variation occurs between individual foods and foods change over time. For example the nutritional content of fresh foods will vary according to growing season, soil and storage conditions. Different cooking methods may be used, affecting, for example, water retention and nutrient content. Furthermore, cooking durations and food preparation methods can affect nutritional content and nutrient availability.

When undertaking dietary analysis researchers must consider the extent to which available databases accommodate foods reportedly consumed by their study participants and compromises may be necessary. For example, seemingly exact information with regard to energy or nutrient content may be available for a given brand of food from the manufacturer. However, analysis is unlikely to have been conducted with the same rigour as that undertaken by McCance and colleagues. Furthermore, current food labelling regulations mean that manufacturers are not legally required to provide full nutritional composition data. Spot checks on manufacturers' food label data have reported inaccuracies and researchers may be wise to base their analyses on a representative sample rather than on manufacturers' less accurate data.[522]

4.5.3.5 Adjustment errors

Body size is also reported to influence dietary intake assessments where overweight and obesity are commonly associated with underreporting of energy intake.[523-525] For example, in studies using the doubly labelled water method for estimation of energy intake, obese women underreported energy intake compared with lean counterparts.[526] Evidence of under-reporting in obese individuals also comes from a study among Canadian adolescents where females were more likely to under-report compared with males and under-reporting increased with increasing weight status for both sexes.[527] It is also important to consider the energy contribution of foods. For example, an individual's intake of

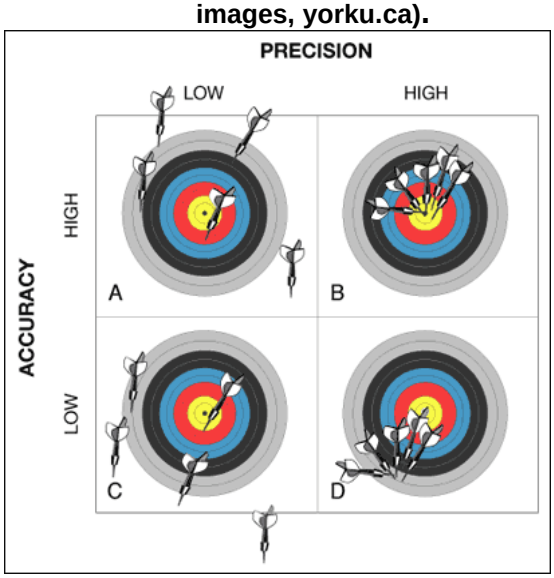
a given nutrient may appear high or low unless adjusted for the total energy consumed.

No method has yet been devised that can measure usual intake with absolute accuracy and therefore no method can be truly validated. Different methods can, however, be compared and the weighed inventory method is considered the best reference for this purpose.[528] However, for reasons discussed above, collection of weighed food records is not possible in many studies. Where it is not feasible to use the weighed inventory method, alternative methods can be used. Possible limitations of these have been discussed above. However, steps can be taken to minimise bias by using validated dietary assessment methods.

4.6 Validity of dietary assessment methods

The errors discussed above affect the validity of dietary assessment. Validity encompasses elements of accuracy and precision. Accuracy describes how close a measured value is to its actual or true value. Measurement of energy intake can be used as an example to illustrate this. Accuracy of energy intake estimates from dietary reports can be assessed using doubly-labelled water, which provides a precise measure of metabolisable energy intake among free-living individuals, as a reference.[529] Precision relates to its ability to reproduce results i.e. the consistency of the questionnaire to measure intake in the same individual at different time points. With regard to precision, it is important to appreciate that absolute reproducibility is unlikely to be achieved due to the risk of bias. For example, dietary intake is likely to show true variation at different time points.

Figure 4-4 Illustration of precision and accuracy as independent concepts. Each column (A, C and B, D) has the same precision and each row (A, B and C, D) has the same accuracy (google images, yorku.ca).



Validity of a dietary assessment tool can be assessed by comparison against a superior measure of intake either from an alternative dietary assessment instrument or using biomarkers of intake. However, it is well recognised that no perfect standards exist and so dietary assessment cannot be performed with one hundred percent accuracy. Therefore, the exact truth cannot be known and validity can only be assessed relatively. It has often been stated that there is no gold standard that can be used to validate prospective methods of dietary assessment in free living people.[459] This led to a quest for alternative standards against which dietary assessment tools could be compared. Over the last few decades several biomarkers have been identified that provide a quantitative measure of dietary intake that does not rely on reports of food consumption.[459, 530]

4.6.1 Biochemical Markers of Dietary Intake

The advantage of measuring biomarkers of intake is that they do not rely on subjects' abilities to recall intake or on their compliance with recording methods. Furthermore they are less susceptible to measurement error **(Section 4.5.3)**. Bingham and colleagues conducted an extensive validation study to examine the validity of dietary assessment methods for use in the UK arm of the European Prospective Investigation of Cancer (EPIC, **Section 4.7.1**). Validity was

assessed by comparing intake of nitrogen, potassium and carotene from 7 day weighed records with three biochemical markers of intake: 1) 24-hour urinary nitrogen; 2) 24-hour urinary potassium; and 3) serum carotenoids respectively. Completeness of 24 hour urine collections was verified against the *P*-aminobenzoic acid (PABA) check technique.[531] This showed that ≥16 days of weighed records and eight 24-hour urine collections were needed to assess dietary intake. The protocol was then used to assess the validity of a range of dietary assessment methods. Three methods were considered suitable for use in EPIC – a 7-day food diary, a FFQ and a 24-hour recall.

Several biomarkers have been investigated to assess their use as indicators of dietary intake. Alkylresorcinols, phenolic lipids present in the outer parts of wheat and rye grains, have been proposed as specific dietary biomarkers of whole-grain intakes and concentrations in plasma, urine and adipose tissue used as dietary biomarkers of wholegrain intake in several small intervention studies.[280, 532] Measurement of urinary sodium via 24-hour urine collections can be used to validate dietary sodium dietary[533] and 24-hour urinary iodine excretion has been reported as a useful biomarker for validating dietary milk and other dairy foods in pregnant Norwegian women.[534]

Folate, vitamin B_{12}, iron, and zinc are particularly important nutrients for women of childbearing age. Fayet and colleagues (2011) assessed the relative validity of a FFQ by comparing estimated dietary intake with biomarkers of folate, vitamin B_{12}, iron, and zinc.[535] Significant correlations between biomarker and nutrient intakes were found for folate (r = 0.37 [P < 0.01]) and vitamin B_{12} (r = 0.27 [P < 0.01]). The validity of the Northern Sweden FFQ for assessing dietary intakes of folate, vitamin B_{12}, riboflavin and vitamin B_6 was also assessed by comparison with plasma levels of these nutrients.[536] Nutrient intakes estimated by the FFQ were moderately correlated with plasma levels (range: 0.13-0.33), demonstrating similar validity to that of FFQs used in other studies.[537, 538, 539, 540]

Biomarkers of fatty acid status provide useful objective measurements against which dietary assessment instruments can be compared. Although biomarkers do not reflect absolute intake, measurement of fatty acids in various biological

samples reflects proportional intake of fatty acids.[541] Suitable biomarker tissues include adipocytes,[542] plasma phospholipids or cholesterol esters[543] and erythrocytes (RBC).[544] Plasma fatty acids reflect recent intake (over 3-4 days) whereas adipocyte and RBC concentrations provide information about long term fatty acid intake (over several months). A major disadvantage of adipocyte fatty acid measurement is that sample collection is invasive and painful and therefore often not acceptable to study participants. As a result plasma or RBC concentrations are most commonly assessed.

Both plasma phospholipids and cholesterol ester fatty acid concentrations correlate reasonably well with dietary estimates from FFQs. Correlation coefficients between dietary and plasma PUFA (expressed as % total FAs) for plasma phospholipids and cholesterol esters were r = 0.25 and r = 0.31 respectively.[545] Confirmation that plasma and erythrocyte FA's reflect recent and longer term dietary intake respectively and therefore serve as biomarkers comes from intervention studies where plasma or RBC FA concentrations were significantly higher in supplemented versus non-supplemented individuals.[546] [253]

Harris and Thomas (2009) suggest that RBCs may be the preferred tissue for assessing *n-3* LC-PUFA status.[544] In a study assessing biological variability of RBC and plasma FA concentrations within subject variability was lowest in RBCs (4.1% ± 1.9 vs. 15.9 ± 6.4%).

4.7 Studies including dietary assessments of *n-3* LC-PUFA

Observational and experimental studies investigating relationships between *n-3* LC-PUFA and vascular health have used various dietary intake assessment tools to track compliance with interventions or predict disease risk. Most investigators have adapted comprehensive tools that have been used previously for example in national surveys or large scale epidemiological studies.

4.7.1 The EPIC Food Frequency Questionnaire

The European Prospective Investigation into Cancer and Nutrition (EPIC) was designed to investigate the relationships between diet, nutritional status, lifestyle

and environmental factors and the incidence of cancer and other chronic diseases. This large-scale study recruited over half a million (520,000) people in ten European countries: Denmark, France, Germany, Greece, Italy, The Netherlands, Norway, Spain, Sweden and the United Kingdom.[547] The methods used to assess diet in such large studies must be of good technical quality, feasible and acceptable to local populations. Extensive methodological studies were carried out to test the validity of dietary questionnaires for proposed use in EPIC. Three dietary methods were adopted: a self-completed dietary questionnaire, an interview-based dietary questionnaire or a FFQ, where the participants estimate their average frequency of intake of a list of foods over the previous 12 months) combined with a seven-day record.[530] In the present RCT the EPIC Norfolk FFQ was used to monitor compliance and assess relationships between dietary exposures and risk of primary CVD.

4.7.1.1 The EPIC FFQ for assessment of n-3 fatty acid intake

The validity of the EPIC FFQ for assessment of *n-3* LC-PUFA was evaluated in a large study of 3,000 EPIC participants. Plasma phospholipid *n-3* PUFA concentrations were used as biomarkers of fish intakes and compared with estimates from 4 dietary assessment methods (FFQ, health and lifestyle questionnaire, 7-day diary, and first-day recall from the 7-day diary). Correlations between fish consumed and plasma phospholipid *n-3* PUFAs were not substantially different between the 4 dietary methods.[548]

4.8 Dietary Assessment in Studies of *n-3* LC-PUFA and vascular health

Dietary *n-3* LC-PUFA intake has been assessed only in some studies investigating their relationship with vascular health.[188, 237, 242, 243, 245, 248, 252, 253, 382, 428, 429, 549, 550] Details of studies with summaries of dietary assessment methodologies can be found in Appendix 1-2, tables 2-2 and 2-3. To the author's knowledge, no study has yet been published where a validated method for assessment of *n-3* LC-PUFA in the specific study population was used or developed. Methods available for evaluating intake of *n-3* LC-PUFA and specifically those found mainly in oily fish (EPA, DHA) are scarce.

Various FFQs have been validated in the UK population against a range of biochemical markers of intake and/or reference dietary assessment methods.[459, 537] FFQs that cover all foodstuffs can be long and complicated, imposing a considerable burden on participants and therefore compromising reliability of the method. Compressing the questionnaire, by grouping foods together, can lead to a loss of detailed information and underestimate intake of specific nutrients. This is an example of random error discussed earlier **(Section 4.5.3)**. Where measurement of a single or few nutrients is required a shorter FFQ focussing on those nutrients may be more practical.

A Pubmed review identified 20 published reports of studies that aimed to validate a method for dietary assessment of *n-3* LC-PUFA. These have been conducted in various locations: 5 from Scandinavia, 4 each from Australia and Japan, 3 from North America and one each from France, Spain and the UK. Thirteen studies assessed FFQ either against a reference dietary assessment method[501, 551, 552] or a biomarker **(Table 4.2)**.[546, 553-560] All studies that compared dietary intake assessments with an objective biomarker reported moderate to good correlations: EPA (r = 0.21-0.58), DHA (r = 0.32-0.53).

4.9 Conclusions

FFQs are a feasible method for dietary assessment of *n-3* LC-PUFA. There is no validated FFQ for assessment of *n-3* LC-PUFA in the UK where the population and dietary habits differ. Such an instrument, if shown to be valid, would provide a much needed practical tool that is both inexpensive and less arduous for researchers and clinicians to use in studies and clinical practice and poses the least burden on study participants. A major aim of the present study was therefore to develop a FFQ for assessment of *n-3* LC-PUFA intake in the UK.

Table 4-1 Dietary Intake Assessment Methods

Dietary Assessment Method	Brief description	Advantages	Disadvantages
Prospective methods			
Weighed records	Participants are taught to weigh and record the food and its weight immediately before eating. Leftover food is also weighed.	Accurate. Does not rely on memory.	Expensive, time consuming. May lead to change in usual diet. Requires high degree of participant co-operation. Places large burden on investigators and participants.
Estimated records	Subjects are taught to keep records using standard household measures.	Simpler and less demanding. Allows rapid, low-cost assessment. Less likely to lead to dietary changes.	Lower accuracy when compared with weighed records.
Observed weighed records	Involves trained researcher to visit subject at meal times and weigh food to be consumed. The researcher then returns to weigh leftover food.	Useful where subjects are unable to keep records for themselves e.g. children.	Demanding for subjects and investigators. Expensive.
Retrospective methods			
Records combined with direct analysis (duplicate portion)	Food composites collected after completion of dietary surveys. Complete duplicate portions of all foods are collected prior to consumption over a whole day. Aliquots of foods collected during dietary surveys are analysed.	Necessary when accurate values in food composition tables are not available.	Very demanding and expensive. Difficult to obtain co-operation from participants.

Dietary Assessment Method	Brief description	Advantages	Disadvantages
24-hour recall (single or multiple pass)	Dietary intake data collected by means of interview when individuals are asked to describe food intake over the previous 24 hours or for the previous day. Multiple pass (recalls for more than one day) give better estimate of habitual intake.	Quick and simple to perform. Places minimal burden on subjects.	Difficulties in recalling intake accurately. Problems with describing foods and portion sizes. Requires trained observer. Multiples are required to describe habitual intake.
Dietary history	Assesses an individual's food intake and usual meal pattern. Information is best obtained by a nutritionist or relevantly trained person.	Provides information about diet spanning long time period. Requires minimal input from subjects.	Focuses on regular patterns, therefore irregularities may be overlooked. Highly subjective.
Food Frequency	Estimates frequency of food consumption over a specified time period.	Cheap, simple and quick method. Subjects can self complete. Coding allows rapid data entry. Provides information about habitual dietary intake. That can be used to identify dietary patterns.	Development of questionnaire is time consuming. Data collected may not be comprehensive. Not fully quantitative – conversion to nutrient or food group data requires extra work.

With the exception of dietary history and food frequency questionnaires considerable time would be required for accurate coding in the above methods. This would place considerable burden on investigators and cost implications for research.

Table 4-2 Crude and adjusted correlations for dietary methods versus reference methods in studies of *n-3* LC-PUFA dietary assessment

Author, year (location)	n	Dietary assessment method validated	Reference methods	Crude	Energy Adjusted
Andersen, 1998. (Norway).[546]	119	FFQ	Subcutaneous Fat	EPA: 0.52*† DHA: 0.49*†	
Marckman, 1995. (Denmark).[561]	24	3 x 7d weighed food records	Subcutaneous fat	EPA: 0.40† DHA: 0.66†	
Sullivan, 2006. (Australia).[553]	53	FFQ	As % Total Erythrocyte membrane Fatty Acids	EPA: 0.40* † DHA: 0.39*†	
Hodge, 2007. (Australia).[554]	4439	FFQ	Plasma Phospholipid Fatty Acids	EPA: 0.18* DHA: 0.4*†	EPA: 0.40* DHA: 0.78*
McNaughton, 2007. (Australia).[555]	43	FFQ	Plasma Phospholipid Fatty Acids	EPA: 0.21* DHA: 0.32*†	
		Weighed Record		EPA: 0.22* DHA: 0.43*†	
Hjartaker, 1997. (Norway).[556]	234	FFQ	Serum Phospholipid Fatty Acids	EPA: 0.58* DHA: 0.53*†	
Andersen, 1999. (Norway).[546]	135	FFQ	Serum Total Fatty Acids	EPA: 0.51*† DHA: 0.38*†	
Sasaki, 2000. (Japan).[562]	42/44≠	DHQ	Serum Phospholipid Fatty Acids	EPA: 0.64/0.61≠† DHA: 0.46/0.46≠†	EPA: 0.64/0.65 ≠ DHA: 0.44 †/0.59†
Kuriki, 2003. (Japan). [563]	94	7-d weighed record	Plasma Total Fatty Acids	Adjusted data only	EPA: 0.57† DHA: 0.57 †

Author, year (location)	n	Dietary assessment method validated	Reference methods	Crude	Energy Adjusted
Kobayashi, 2001. (Japan). [564]	87	7-d weighed record	Serum Phospholipid Fatty Acids	EPA: 0.75† DHA: 0.50†	EPA: 0.89†‡ DHA: 0.61†‡
Tokudome, 2001 (Japan).[551]	79	FFQ	28-d weighed record	EPA: 0.25/0.26†† DHA: 0.26/0/29††	EPA: 0.39/0.32†† DHA: 0.43/0.31††
Paalenen, 2006. (Finland).[501]	294	FFQ	3-d weighed record		Total *n-3* LC-PUFA: 0.35/0.20 ≠
Broadfield, 2003. (UK). [552]	31	FFQ	3-d weighed record	EPA: 0.59‡ DHA: 0.55‡†	EPA: 0.50* DHA: 0.37*
Astorg, 2008. (France). [565]	276/257≠	Multiple 24-hour recalls	Serum total Fatty Acids	EPA: 0.24/0.27‡≠ DHA: 0.23/0.29‡≠	
London; 1991. (USA).[557]	115	FFQ	Adipose Fatty Acids	Total Marine FA: 0.48*†	
Sublette, 2011. (USA).[558]	61	FFQ	Plasma total Fatty Acids	EPA: 0.38*† DHA: 0.50* †	
Mina, 2007. (Australia).[559]	91	FFQ	As % Total Erythrocyte membrane Fatty Acids	EPA: 0.47* † DHA: 0.0.33* †	
Amiano, 2001 (Spain). [566]	120	Diet History	Serum Phospholipid Cholesterol Ester Fatty Acids	EPA: 0.52*†‡ DHA:0.62 EPA: 0.55*†‡ DHA:0.50	
Lucas, 2009. (Canada).[567]	65	FFQ	As % Total Erythrocyte membrane Fatty Acids	EPA+DHA: 0.42‡ † From marine foods.	

* Spearman correlation, †Significant at $P < 0.001$, ‡Pearson correlation, † Significant at $P < 0.05$, ≠ Men/women, †† Pearson/Spearman correlation

Chapter 5

General Methods

By three methods we may learn wisdom: First, by reflection, which is noblest; Second, by imitation, which is easiest; and third by experience, which is the bitterest (Confucius).

Section 2: Methods

5.1 Introduction

This PhD project was part of a randomized controlled trial designed to investigate the effects of supplementation with the *n-3* LC-PUFA, DHA, on vascular function and risk factors for CVD in healthy young adults. The primary outcome was endothelial dysfunction, an early stage in the atherosclerotic process.

Previously, our research group assessed endothelial function using FMD in young adults aged 20-28 and showed an association between FMD and both plasma and red cell DHA concentrations.[568] A similar association was seen for CVD risk factors including plasma glucose, insulin and TG concentrations. This suggests that higher *n-3* LC-PUFA concentrations may have a protective role against endothelial dysfunction in young healthy adults who are unlikely to have established CVD. Plasma DHA concentration correlated with dietary fish intake, and a dose-response association between DHA concentration and FMD was reported that was independent of the lipid profile. This suggests that dietary supplementation with *n-3* LC-PUFA could be causally related to improved FMD in this population. Furthermore, associations of FMD with *n-3* LC-PUFA levels in the normal dietary range suggest that an improvement in endothelial dysfunction could be achieved by moderate increases in *n-3* LC-PUFA intake.

Evidence from epidemiological studies suggests that high fish consumption is associated with a lower risk of CVD **(See Chapter 2, Section 2.4)**. This association is likely to be mediated by the high levels of the *n-3* LC-PUFAs EPA and DHA found in highest concentrations in fish oil. An inverse correlation

between the dietary intake of these fatty acids and CVD has been shown in most of these studies.[180, 235, 243, 256, 270, 275, 299, 340, 353, 386, 430, 434, 568-578]

Despite the strong epidemiological data, a causal association between *n-3* LC-PUFA intake and the prevention of CVD has not been established. Although randomized intervention trials have shown beneficial effects of *n-3* LC-PUFA supplementation, these trials have focused on secondary prevention and few studies have investigated the influence of *n-3* LC-PUFA on the primary prevention of atherosclerosis or coronary artery disease. However, preliminary evidence now suggests that *n-3* LC-PUFA may have a favourable effect on endothelial function and this could be one mechanism for their benefits in both the primary and secondary prevention of coronary artery disease.

5.1.1 Hypotheses

The aims of the RCT were to test the hypotheses detailed below in sections 5.1.1.1 and 5.1.1.2.

5.1.1.1 RCT Primary Hypothesis:

Hypothesis 1: *n-3* LC-PUFA supplementation improves vascular function (flow-mediated endothelial dependent dilation, (FMD)) in healthy young adults.

5.1.1.2 Secondary Hypotheses:

Hypothesis 2:*n-3* LC-PUFA supplementation improves CVD risk factors including blood pressure, fasting insulin, glucose, CRP and lipids.

Hypothesis 3: Effects of *n-3* LC-PUFA supplementation on vascular function are influenced by gender and lifestyle factors, including smoking.

Hypothesis 4: The magnitude of *n-3* LC-PUFA effects differ according to supplement dose.

The aims of this PhD thesis were to examine relationships of diet with vascular structure and function in healthy young adults. The study was conducted in parallel with the RCT which provided a convenience sample.

In **Chapter 2**, a comprehensive review found strong evidence for a beneficial effect of 'prudent' or 'healthy' dietary patterns on primary prevention of CVD. Evidence for the role of specific dietary components within such patterns suggested fish and *n-3* LC-PUFA play a major role in the protective effect. The following hypotheses were formed on the basis of current evidence for the relationship of diet and primary CVD.

Hypothesis 1: Dietary patterns are associated with vascular structure and function in young healthy adults.

Hypothesis 2: Components within dietary patterns, specifically *n-3* LC-PUFA are associated with vascular structure and function.

Evidence suggests that associations of diet with vascular function may operate through classical CVD risk factors such as blood pressure, lipid concentrations, insulin resistance and obesity. Therefore the following hypotheses were formulated:

Hypothesis 3: Dietary patterns are associated with CVD risk factors in young healthy adults.

Hypothesis 4: Components within dietary patterns, specifically *n-3* LC-PUFA are associated with CVD risk factors in young healthy adults

Reliable assessment of *n-3* LC-PUFA intake is essential in studies investigating relationships between fatty acids and health outcomes. Therefore **a secondary**

aim of this PhD was to develop and validate a FFQ (FishFQ) designed specifically to assess *n-3* LC-PUFA intake. A FFQ designed to collect information on usual intake of foods rich in DHA and EPA was developed and tested as part of the RCT. This formed the basis for the following additional hypotheses:

Hypothesis 5: A FishFQ specifically designed to assess dietary *n-3* LC-PUFA has acceptable validity when compared against a reference dietary assessment method (multiple 24-hour dietary recalls) and biomarkers of *n-3* LC-PUFA intake using the method of triads.

Hypothesis 6: The FishFQ is able to accurately classify individuals according to thirds of *n-3* LC-PUFA intake.

Hypothesis 7: The FishFQ is reliable and results can be reproduced at different time points.

5.2 Study Design

The study was a double-blind, parallel group, placebo-controlled randomized trial conducted at the Medical Research Council (MRC) Childhood Nutrition Centre (CNRC), University College London (UCL) Institute of Child Health (ICH), London, between 2003 and 2008. Participants were randomly assigned to DHA supplementation or control groups using a randomisation list generated by an independent statistician and held at a collaborating centre (Martek, Biosciences corporation, Maryland, USA). The assigned dietary group was allocated using numbered, sealed, and opaque envelopes and all participants and research staff were blind to the dietary assignment. FMD and other study outcomes were measured during a 1-day visit to the research centre before and after 4 months of dietary intervention. All subjects were asked to participate in post-intervention measurements irrespective of whether they complied with the study protocol (intention to treat).

5.2.1 Subjects

Participants included members of a population previously studied by our research group who were now aged 28–34 years. These subjects were originally identified by sending a letter of invitation to a random sample of individuals born at the Cambridge Maternity Hospital between 1969 and 1975. The group was chosen because concise records of birth and growth had been kept. A total of 326 (157 males and 169 females) subjects were potentially available. Members of this cohort were traced by sending a letter to their GP **(Appendix 2-1).** A letter of invitation explaining the study was sent to these previous and potential participants **(Appendix 2-2)**. Additional younger adult volunteers were recruited from students, staff and their friends and families at UCL. An "all users email" was sent through the UCL email network and posters were placed on notice boards in UCL buildings **(Appendix 2-3)**. An article, written by myself, was placed in the professional journal of the British Dietetic Association *'Dietetics Today'* inviting members to participate. Further participants were recruited by word of mouth.

Individuals who were aged 18-37 years, clinically well at the clinic visit, and free from chronic disease likely to affect FMD (e.g. insulin dependent diabetes), were eligible. Those who were pregnant, on unusual diets, or taking regular medication or *n-3* PUFA supplements were excluded. This initial contact was followed by a telephone call for a full discussion of any issues raised. During this call the procedure for providing informed consent was explained and participants were given ample time (at least two weeks) to consider whether they would like to take part in the study. A patient information sheet with a fuller explanation of study procedures was sent to potential participants **(Appendix 2-4)**.

5.2.2 Sample size

The minimum projected sample size was 256 subjects (128 each in *n-3* LC-PUFA supplemented and placebo groups). The sample size was adequate to detect a 0.35 standard deviation (SD) difference in FMD between randomized groups at 5% significance and with 80% power. The study was therefore conservatively powered, as most risk factors for endothelial dysfunction are associated with a

0.5 to 1 SD reduction in FMD[579] and interventions to improve endothelial function (e.g. exercise training) have often produced a greater than 1 SD improvement in FMD[580]. Furthermore, a previous study by our group suggested that a 1% increase in plasma DHA level was associated with a 0.5 SD increase in FMD[568]. Supplementation was set at 1.6g/day – a level that was considered more than adequate to produce a 1% improvement in red cell *n-3* LC-PUFA concentration. Supplementation for 4 weeks with 0.9g/day *n-3* PUFA has been shown to increase red cell membrane *n-3* fatty acid concentration by 0.76%.[581]

5.2.3 Inclusion Criteria

Inclusion criteria were: aged 18-37 years, willing to participate, clinically well at the first clinic visit, and no clinical diagnosis of atherosclerosis. Exclusion criteria were: women who were pregnant at the initial visit, chronic disease likely to affect endothelial function (e.g. insulin dependent diabetes), adherence to unusual diets and currently taking *n-3* supplements.

5.2.4 Ethical Approval

Ethical approval was applied for and granted from the Metropolitan Multi Research Ethics Committee, Lewisham, London. The trial was registered on the International Standard Randomised Controlled Trial Register as follows: ISRCTN no. 19987575 (http://www.controlled-trials.com).

5.2.5 Participant flow through randomized trial

Participants attended for assessment at the Childhood Nutrition Research Centre (CNRC), Institute of Child Health (ICH), University College London (UCL) on two occasions: visit 1, at which baseline measurements were taken, preceded the intervention and visit 2, where measurements were repeated following supplementation, was arranged as close to the end of the 16 week intervention period as possible. At the first visit, the study was explained to the subjects by one of four CNRC researchers who were fully trained in good clinical practice (GCP) and research governance. Informed consent was provided by all participants to take part in the study and additionally, if willing, participants gave consent to provide a blood sample **(Appendix 2-5 & 2-6)**.

The study was conducted in CNRC and Department of Vascular Physiology (VP), at UCL/ICH. Travelling and other expenses were refunded and overnight accommodation provided where necessary. Participants were given £50 on the day of their last clinic visit, as a thank you for their time and inconvenience. Flow charts outlining the study protocol and the experimental flow of subjects through the trial can be found in **Appendix 2-7**.

5.2.6 Intervention

Subjects were randomized to receive either 1.6g/day DHA or placebo (olive oil). For comparison, the level of supplementation was approximately twice that used in a study (0.9g/day fish oil) that showed a 29% reduction in mortality in a fish supplemented group.[195] However, the level of supplementation was half that used in several other studies and in the trial on the effects of *n-3* LC-PUFA on coronary bypass graft patency.[582] The level of supplementation was lower than that safely used in previous studies and that recommended for Maxepa® capsules in the British National Formulary. This level of *n-3* LC-PUFA intake is also equivalent to approximately 1 large portion of oily fish (e.g. herring or salmon) per day and is therefore within a plausible dietary range for the population. Supplementation of less than 3g of *n-3* LC-PUFA per day has been categorized as GRAS (Generally Regarded As Safe) by the US Federal Drug Authority.[190]

5.2.6.1 Supplement and placebo details

The supplement took the form of 8 x 500mg gelatin capsules containing DHA rich oil providing 200mg DHA as triglycerides per capsule (DHA-S). More specifically, DHA-S oil is a mixed triglyceride, meaning that the oil contains a mixture of triglyceride molecules, most of which have at least one DHA molecule. As in most natural food sources of DHA, the DHA in DHA-S oil is found primarily at the *sn-2* position on the glycerol backbone of a triglyceride molecule, with the remainder at *sn-1* and *sn-3* positions **(Chapter 3, Figure 3-2)**. The source of this oil was the microalgae Schizochytrium *sp*. Approval for the use of this DHA rich oil as a novel food was recommended in the UK by the Advisory Committee on Novel Foods. Subsequently approval was obtained from the European

Commission as a novel food ingredient under EC regulation no. 258/97.[583] Placebo capsules took the same form and contained 500mg olive oil per capsule. Eight capsules were taken daily providing a total of 4g olive oil/day. All capsules were provided by Martek Biosciences Corporation (Maryland, US, now DSM Nutritional Lipids).

DHA is a 22-carbon omega-3 fatty acid with six double bonds (also referred to as *n-3*), and is designated 22:6*n-3* or 22:6 *omega-3*. It is considered an omega-3 or *n-3* fatty acid because its first double bond occurs on the third carbon from the methyl or "omega" end of the molecule. DHA is highly lipophilic and, due to the large number of double bonds, has a low melting temperature and is a liquid (oil) rather than solid (wax) at room temperature.

5.2.6.2 Compliance Monitoring

Compliance was monitored with regular phone calls and/or text messages sent by a member of the research team. Attempts were made to contact each participant weekly. During these calls, participants were asked to answer investigator defined questions relating to the participant's health during the preceding seven days or since the previous contact, where the interim period exceeded seven days **(Appendix 2-8)**. During compliance monitoring telephone contacts, researchers requested participants to provide a 24-hour recall of dietary intake. For each participant, the aim was to collect 7 dietary recalls during the intervention period; these should ideally be representative of typical week and weekend days. Full details of the design and development of the methods for collection of 24-hour dietary recall can be found in **Chapter 6, Section 6.4.1** Compliance was further assessed by counting remaining capsules at the end of the intervention period.

5.2.6.3 Biological Markers of *n*-3 Consumption

Assessment of erythrocyte membrane concentrations of *n-3* PUFA was carried out prior to supplementation to assess fatty acid status. Measurements were repeated post-supplementation to provide objective evidence of compliance with

supplementation and for assessment of dietary changes in fatty acid status that would reflect consumption. Blood was obtained by venepuncture between 0900 and 1100 after an overnight fast. All procedures were carried out by trained staff in accordance with the study protocol and CNRC standard operating procedures (SOP). Red cell membranes were prepared on the day of collection and all samples stored at -70^{o} C prior to analysis. Briefly, whole blood was prepared in lithium-heparin with phosphate buffered saline and centrifuged to remove the buffy coat. Red cells were washed 3 times with saline, by re-suspension and centrifuging. The samples were thawed only once immediately before analysis. Red cell ghosts were analysed for *n-3* fatty acid concentrations by Omegametrix using the methodologies of Professor Clemens von Schacky. (http://www.omegametrix.eu). Individual fatty acids were measured and reported as a percentage of total red cell membrane fatty acids.

5.2.7 Dietary assessment

The EPIC FFQ was used to assess participants' usual diet during the year prior to enrolment. The FFQ was administered again at the end of the supplementation period to assess diet during the intervention period to ascertain whether there were any systematic changes in diet as a consequence of participating in the study.

A separate FFQ was designed specifically for assessment of *n-3* LC-PUFA intake (FishFQ). This was to be validated in the RCT participants. The FishFQ was administered before and after the RCT intervention period. Full details of administration of the EPIC and FishFQs are given in **Chapter 6, Section 6.2**. The relative validity of the FishFQ was assessed by comparison with erythrocyte membrane concentrations of *n-3* LC-PUFA and a reference dietary assessment method - multiple 24-hour recalls of dietary intake. A full description of the development and use of dietary assessment methods is given in **Chapter 6**.

5.3 Outcome measures

All measurements were made by investigators who were blind to the supplementation status of the subjects. The measurements were carried out at baseline and after 4 months supplementation.

5.3.1 Vascular Measurements

All vascular studies were performed in a temperature controlled (22^{o} C to 26^{o} C) vascular laboratory by one of two trained operators. Subjects were rested supine for 10 minutes before measurements.

5.3.1.1 Brachial Artery Flow Mediated Dilatation

The principal trial outcome was FMD of the brachial artery. Participants were asked to avoid foods with high fat content during the morning preceding the scan as these have been reported to affect FMD.[198] In addition, they were asked to refrain from ingesting substances that may affect flow-mediated vascular reactivity, including caffeine[584], tobacco[585] and vitamin C.[284]

Participants were asked to lie flat on an investigation couch, with the top half of their body as close to the scanning table as possible. Long-sleeved clothing was removed to expose the upper arm. The height of the couch was adjusted to achieve alignment with an adjacent arm rest. A pneumatic FMD cuff was placed around the forearm immediately below the elbow (1cm distally to the medial epicondyle, **Figure 5-1**).

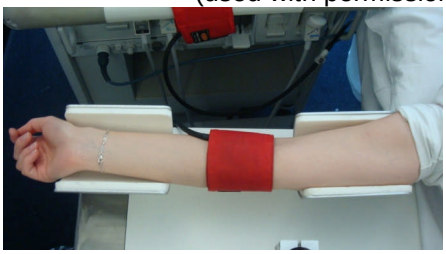
Room and skin temperature may influence blood vessel diameter.[39] Therefore room temperature was recorded to ensure the optimal range of 24–26° C. Similarly skin temperature was recorded and if lower than 31° C, a heat pack was applied to raise the temperature. During measurement of FMD it is essential to ensure that participants are both comfortable and warm as unnecessary movements during the study could affect image quality.

Electrocardiogram (ECG) leads for measurement of cardiac activity were positioned below the left and right clavicles and one approximately 5 cm right of

the umbilicus. A blood pressure cuff was placed on the left arm. The right arm was placed in an arm rest at an angle of 70–90 degrees to the body **(Figure 5-2)**. The brachial artery was imaged in longitudinal section, 5-10 cm above the elbow, using a 5-10 MHz linear array transducer and an Acuson 128XP/10 system (Accuson, Aspen, Siemens, Malvern, Pennsylvania, USA). The transducer was fixed using a stereotactic clamp and fine position adjustments made when necessary using micrometer screws.

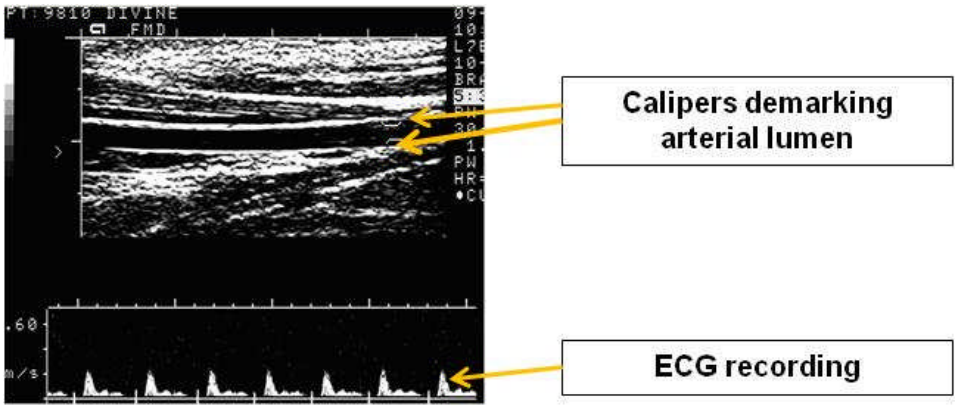
Figure 5-1: Medial epicondyle reference point for cuff positioning in FMD
(Google images, copyright Mayo Clinic.com)

Figure 5-2: Correct arm and pneumatic cuff positioning in FMD
(used with permission, Vascular Physiology Department, UCL/ICH)



Room lights were dimmed to create a relaxed environment for the scan. Participants were requested to remain as still as possible throughout the procedure. Ultrasound gel was placed on the crown of the biceps brachial muscle. The ultrasound probe was positioned on the gel and manipulated until a satisfactory image was obtained of a brachial artery section **(Figure 5-3)**.

Figure 5-3: Brachial artery image obtained using Doppler ultrasound



Brachial artery diameter was measured with edge detection software (Brachial Tools, Iowa City, Iowa). Ultrasound calipers were used to mark the boundaries of the arterial lumen as shown above **(Figure 5.3)**. ECG gated end diastolic B-mode images were digitized and stored off line sequentially every 3 seconds throughout the scan procedure for later arterial diameter measurements. Blood pressure was monitored using an automated oscillometric device (Accuson, Aspen, Siemens, Malvern, Pennsylvania, USA) before and after FMD measurement and resting heart rate recorded using the three lead ECG linked to the Accuson 128XP/10 ultrasound machine.

The pneumatic cuff was inflated around the forearm to 300 mm Hg for 5 minutes followed by rapid deflation causing a large increase in blood flow, (reactive hyperemia). Participants were asked to remain still for the duration of the scan, which took 11 minutes in total (1 minute resting, 5 minutes cuff inflation and 5 minutes post cuff deflation). The resting and post hyperemic blood flow velocities were measured in the centre of the imaged artery using pulsed wave Doppler ultrasound.

Reactive hyperemia was calculated from the maximal flow within the first 15 seconds after deflation of the pneumatic cuff, relative to baseline flow. FMD was expressed as the absolute maximal change between pre- and post-hyperemic brachial artery diameter adjusted for pre-hyperemic diameter using regression analysis[586] and as a percentage change from baseline arterial diameter. Absolute change in diameter was chosen as the primary outcome because it is

independent of baseline arterial diameter, which contributes to sex differences in FMD expressed as a percentage change. The reproducibility and detailed methodology for measuring flow mediated endothelial dilation has been previously described.[586-588]

5.3.1.2 Brachial Arterial Distensibility

Arterial distension was measured in the same arterial section selected for FMD assessment. The ultra-sound probe was aligned with the M-mode (motion-mode) cursor positioned at right angles to the arterial lumen over the clearest defined section of the artery on the B-mode image. A five-second segment of the radio frequency signal was recorded by a separate Wall Tracking System (Ingenious Medical Systems, Arnhem, Netherlands) at a rate of 800 Hz (one frame per millisecond) and the initial frame (amplitude waveform) displayed. Two sample volumes were set coinciding with the arterial wall-lumen interfaces and the relative position of the walls within these volumes were measured every 25 milliseconds (around one fifth of the expected time for an upstroke) using a displacement detection algorithm based on the cross-correlation model for corresponding segment of the radio frequency lines. The mean change in diameter between diastole and systole over the five-second period was used as the measure of arterial distension. Three distension measurements were made and an average recorded. Previously reported coefficients of variation for measurements of diameter and distension using this technique are 2-3%.[586] Blood pressure was measured in the left brachial artery using an oscillometric device (as above), at the same time that distensibility was measured in the right arm to provide a representative measure of pulse pressure in the right brachial artery. Arterial distensibility was determined at both the brachial and carotid arteries.

5.3.1.3 Carotid Artery Distensibility

The carotid artery was imaged using B-mode ultrasonography at a region 1 cm proximal to the origin of the bulb of the right and left common carotid artery**.** The transducer was manipulated such that the near wall of the carotid artery was parallel to the transducer footprint and the lumen maximized in the longitudinal

plane. A M-mode recording was made with the M-mode cursor perpendicular to the vessel wall. Three images of each B-mode and M-mode in different directions (anterior, lateral, medial) were recorded and the images digitized and saved on a computer. Brachial blood pressure was recorded at 5 minute intervals throughout the period of ultrasound scanning. The artery diameter changes for assessing carotid distensibility and the distensibility coefficient were analyzed as described previously.[589]

5.3.1.4 Carotid Artery Intima-media Thickness (IMT)

The left common carotid was imaged 1 cm proximal to the bifurcation. Longitudinal images of the far wall intima-media interface (M-line) were clearly defined and recorded on videotape for later analysis. The distance between the leading edge of the intima and the media-adventitia was measured with ultrasonic calipers **(Chapter 1, Figure 1.4)**. Three measurements were taken in both the right and left common carotid and mean IMT calculated as described.[590] Briefly, video frames were digitized using a video frame grabber and assessed with software incorporating an edge-detection algorithm specifically designed for use with ultrasound scanning that enables automatic detection, tracking, and recording of the lumen/intima and media/adventitia interfaces.[591] The distance from the leading edge of the first to the second echogenic line was measured as posterior wall IMT.[46]

5.3.1.5 Pulse Wave Velocity

Pulse wave velocity measures the velocity of the blood pressure pulse waveform.[592] A pressure tonometer (SPC-301 Millar Instruments, Houston, Texas, USA) was used to record the pulse pressure waveform transcutaneously in the underlying artery. This was recorded simultaneously with an ECG signal, which provided an R-timing reference. Pulse pressure recordings were performed consecutively at two superficial artery sites. Integral software was applied to process each set of pulse pressures (Sphygmocor version 7.1, Scanmed, UK). The software uses ECG waveform data to calculate the mean time difference between the R-waves and the pressure waves on a beat-to-beat

basis. Pulse wave velocity is calculated using the mean time difference and arterial path length between the two recording points.

5.3.2 Biochemical and hematological cardiovascular risk factors

Blood was obtained by venepuncture between 0900 and 1100 after an overnight fast. Biochemical and haematolgical risk factors for CVD were measured including fasting insulin, glucose, lipid profile and C-Reactive Protein (CRP). These measurements were carried out in the laboratories of Great Ormond Street Hospital, London according to SOPs. Red cell and plasma concentrations of *n-3* LC-PUFA were determined before and after the intervention using standardized methods in the laboratories of Professor von Schacky (omegametrix) and expressed as a percentage of total fatty acids.

5.3.3 Anthropometry

Height, weight, waist, hip and limb circumferences were measured using standard equipment and protocols.

5.3.3.1 Height, weight and BMI

Height was measured using a portable stadiometer accurate to 0.1 cm (Holtain Instruments Ltd., Crymmych, UK) and weight using electronic scales accurate to 0.1kg (Seca Instruments, Hamburg, Germany). Body mass index (BMI), defined as the individual's body weight divided by the square of his or her height was calculated using Quetelet's formula Body mass (kg) / Height (m) [2. 593]

5.3.3.2 Limb circumferences

Waist, hip and limb circumferences were measured using a non-stretchable, calibrated tape measure (Chasmors, London, UK). All limb circumferences were measured on the left side of the body to achieve standardisation. Procedures were repeated 3 times and the best mean measurement used in analyses.

5.3.4 Body composition

5.3.4.1 Measurement of Skinfold Thickness

Triceps, biceps sub-scapular and supra-iliac skinfolds were measured in duplicate on the left side of the body using skinfold calipers (Holtain Instruments Ltd., Crymmych, UK) and the mean value obtained.

5.3.4.2 Estimation of body fat from skinfold thickness

Percentage body fat was estimated from the sum of skinfold thickness at four sites using prediction equations of Durnin & Womersley (1974) and Siri (1961).[594-596] The equations of Durnin & Wormsley use skinfold thickness measurements to predict body density. Provided that body density is known proportions of lean and fat mass can be estimated using Siri's equation which uses the generic equation first described by Brozek (1963).[597] This equation is based on Archimede's Principle and allows the calculation of lean or fat free mass and fat mass based on assumed densities of these two body compartments where fat is assumed to have a constant density of 0.9kg/l and fat free mass of 1.1kg/l.

W1 = (1/D x (d1 x d2/d2-d1)) – d1/ (d2 – d1)

Where:

W1 = Fraction of fat

d1 = Density of fat

d2 = Density of fat free mass

D = Body density (body density/body volume)

5.3.5 Sociodemographic factors

5.3.5.1 Educational status

Educational status was categorised according to the possession or not of a university degree.

5.3.5.2 Social class

Social class was determined based on the subject's occupation according to the Registrar General's Classification (OPCS, 1991).[598] In this system five basic social classes, recognised by the Office of Population Censuses and Surveys (OPCS), are applied:

I	Professional occupations
II	Managerial and Technical occupations
III	Skilled occupations (N) non-manual (M manual
IV	Partly skilled occupations
V	Unskilled occupations

In the present study a dichotomous division between manual and non-manual workers was applied to allow useful comparisons with previous published studies. Students were classified as non-manual.

5.3.6 Lifestyle factors

5.3.6.1 Smoking practice and alcohol consumption

Data describing lifestyle factors including smoking habits were collected using a short questionnaire **(Appendix 2-9)**. Smoking exposure was verified by measurement of salivary cotinine. Salivary cotinine analyses were conducted in the laboratories of Professor Martin Jarvis, ICRF Health Behaviour Unit, Department of Epidemiology and Public Health, UCL, London. Alcohol consumption was assessed using the same lifestyle questionnaire.

5.3.6.2 Physical Activity Level

Habitual physical activity was assessed using the International Physical Activity Questionnaire (IPAQ) questionnaire.[599] Cardiovascular fitness was assessed using a standardized, height specific, step test before and after the intervention as described by Francis and Feinstein (1991).[600]

5.4 Statistical methods

Initial analyses were conducted on an intention to treat (ITT) basis. Student's *t*-test was used to detect differences in continuous normally distributed data

between *n-3* LC-PUFA supplemented and non-supplemented groups. Chi-squared was used for dichotomous variables.

Secondary analyses include 1) multiple regression analyses to assess differences between randomized groups after adjusting for possible differences in baseline variables with potentially confounding effects (e.g. age, gender, temperature, fasting serum lipids), 2) effects of *n-3* LC-PUFA supplementation in sub-groups such as smokers and 3) adjustment of the primary analyses for factors thought to change with *n-3* LC-PUFA supplementation (such as a potential change in the lipid profile). The distributions of fasting VLDL, triglyceride, insulin, CRP and cotinine concentration and sum of skinfold thickness were right skewed and so were $\log_e$ transformed and then multiplied by 100 prior to analysis.[601] The standard deviation for 100 $\log_e$ transformed data represents the coefficient of variation of the original data, while coefficients represent the percentage difference in absolute FMD per unit change in the independent variable.

A dose-response relation between *n-3* LC-PUFA levels and vascular function was investigated by regressing vascular outcomes against *n-3* PUFA concentration. Multiple regression analysis was used to assess associations between changes in vascular function with change in red cell *n-3* LC-PUFA concentrations following supplementation.

All analyses were conducted in SPSS for Windows (version 18.0; SPSS Inc. Chicago) with statistical significance level at $P < 0.05$. Professor T J Cole at the Institute of Child Health reviewed the statistical methods and sample size calculations.

Chapter 6

Validation and Dietary Assessment Methods

He who knows nothing is closer to the truth than he whose mind is filled with falsehoods and errors.
Thomas Jefferson

6.1 Introduction

In recent years, nutritional scientists have recognized that the complexity of diet and inherent errors in methods that aim to accurately describe the magnitude of intake of individual foods and nutrients preclude wholly accurate assessments using quantitative methods. Qualitative dietary assessment methods may therefore be preferable to quantitative methods (**Chapter 4, section 4.4.2**). Over the past decade dietary pattern analysis has emerged as an alternative or complementary approach to examine the relationship between diet and the risk of chronic diseases. In view of this, the present study aims to describe dietary patterns within this group of young adults residing in the UK. It further aims to investigate relationships of dietary patterns and components within it with study outcomes.

The EPIC FFQ was one chosen dietary assessment tool in this study because it has been validated in a similar UK population **(Chapter 4, Section 4.7.1).** Furthermore, it has been used in previous studies undertaken by our research group, in members of this population at a younger age where relative validity was assessed using red cell folic acid as a biomarker.[602] Permission was sought to adapt the FFQ for use in the current study from the EPIC management committee at the Strangeways Research Laboratory, University of Cambridge for which full approval was given.

When considering specific dietary components within 'prudent' and Mediterranean style dietary patterns strong evidence supports a protective role for *n-3* fatty acids and in particular *n-3* LC-PUFA of which good dietary sources include fish and fish oils. Therefore, it was hypothesized that dietary patterns have an important role in the development of CVD and that *n-3* LC-PUFA, in

particular, may be beneficial to vascular health. A further major aim of this dietary study therefore was to assess relationships between dietary *n-3* LC-PUFA and the earliest signs of CVD. This requires a valid method for dietary assessment of *n-3* LC-PUFA that is currently unavailable in the UK.

Assessment of dietary intake is an essential requirement of all studies investigating relationships between food or nutrient exposure and health outcomes and, as discussed in **Chapter 4, Section 4.3**, reliable dietary assessments should be integral to research protocols because public health recommendations should only be based on findings from studies that include valid measures of nutritional intake. It is also important in clinical practice where accurate dietary assessment is needed to allow practitioners to formulate appropriate nutritional advice that aims to redress nutritional deficiencies or excesses.

Methods available for evaluating *n-3* LC-PUFA are scarce and there is a need for a cost effective, non-invasive method that can be used in research and clinical practice. Any new dietary assessment instrument must be compared to a method that has been shown to have acceptable validity **(Chapter 4, Section 4.6)**. Although there are FFQs available which have been validated amongst the UK population none is available that has been assessed objectively for measuring dietary *n-3* LC-PUFA intake. A major aim of this dietary study therefore was to develop a FFQ for estimating *n-3* LC-PUFA in this healthy young UK population.

Erythrocyte membrane fatty acid concentration was assessed to provide an objective measure of fatty acid status and verify compliance with supplementation during the RCT. This also provided a biomarker of fatty acid intake against which dietary assessment methods could be compared. A further aim of this study was to investigate relationships between dietary *n-3* LC-PUFA, estimated by dietary assessment methods, and fatty acid status, determined by biomarker status, with study outcomes including measures of vascular health and markers of CVD risk

6.2 Dietary Assessment Methods

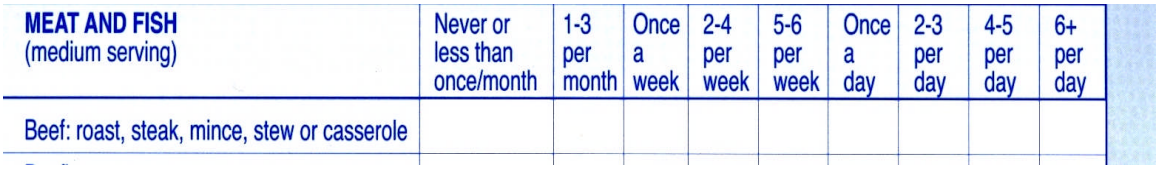
6.2.1 EPIC FFQ

Dietary assessment with the EPIC FFQ was undertaken as part of the RCT to assess possible dietary change that may confound effects of supplementation and/or influence CVD outcomes under study. A further aim was to analyse dietary patterns in the study population to study relationships with CVD outcomes.

The EPIC FFQ is a 10-page A-4 booklet designed to measure a participant's usual food intake during the previous year. An example page is included in **Appendix 3-1**. The FFQ consists of two sections: 1) a main section containing a list of 130 foods and 2) a list of supplementary questions. Participants are asked to indicate their usual frequency of consumption ranging from "never or less than once/month" to "6 times per day" by ticking a box in one of nine frequency categories **(Figure 6-1)**. Example serving sizes are suggested based on standard portions or household measures. For example, one piece of fruit, one slice of bread, a glass of fruit squash, a cup of tea, or a tablespoon of cream.

The second section, at the back of the booklet, comprises 16 additional questions on type and brand of everyday foods and cooking methods. For example, the usual breakfast cereal eaten and type of milk used; whether visible fat was removed from meat before cooking; the type of fat used in cooking and the preferred method e.g. frying, roasting, grilling or baking.

Figure 6-1 Example line entry from EPIC FFQ



MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									

6.2.1.1 Administration of EPIC FFQ

The EPIC FFQ and a newly developed FishFQ **(Section 6.3)** were administered both at baseline and following the 4 month supplementation period. The order in

which questionnaires were given was determined by a randomisation procedure to reduce the risk of bias. The same researcher administered the questionnaires at the two study visits to reduce inter-operator variability. All members of the research team were trained by me, a registered dietitian, in the administration of the FFQ.

The first FFQ was explained to participants in full by a member of the research team. Participants were left to complete the questionnaire alone, but encouraged to ask for assistance if needed from the research team member allocated to their dietary assessment, and who checked for completeness of entry after completion. After completion of the first FFQ participants were given a short break before the 2nd questionnaire was administered.

6.2.1.2 Development of analysis methods for EPIC FFQ

Questionnaires used in EPIC have been designed to work in conjunction with specific supporting programmes. However, commercially available programmes (e.g. CompEat (Carlston/Bengston) and Microdiet (Downlee systems) can also be used effectively. In the present analysis, a dietary analysis instruction manual was produced by me to use when converting dietary intake data collected with the EPIC FFQ to nutrient data using the Microdiet nutritional analysis programme version 2.8.8 **(Appendix 3-2)**.

Research assistants were provided with the dietary analysis instruction manual and fully trained in its application. The manual provided concise instructions for selecting foods based on information given in the EPIC FFQ, including the additional questions. Detailed food lists are given with corresponding food codes drawn from McCance and Widdowson's data on the composition of foods and its supplements, produced by the UK Royal Society of Chemistry **(Chapter 4, Section 4.5.3.4)**. Participants were asked to indicate usual portion size by comparison with photographs in the Ministry of Fishery and Foods (MAFF) photographic atlas of food portion size.[603] Where portion size information was available this was used to estimate the amount in grams usually consumed of a given food. Where no specific portion size information was available the standard portion size was used.[604]

A Microsoft excel template was created for calculation of food amounts in grams consumed daily. The procedure is described in detail in **Appendix 3-2**. Briefly, the amount in grams representative of either a standard or reported portion size was entered to the excel template. A multiplication factor representing frequency of intake was applied to give an average amount of food consumed daily. Food codes were used to locate foods indicated as regularly consumed in the Microdiet nutritional analysis programme. Once the appropriate food was recalled, the amount consumed daily in grams, as calculated using the excel template, was entered to the nutrient database.

6.3 FishFQ

Food frequency questionnaires which have been validated against various biomarkers are available in the UK. However, to my knowledge there is only one published validation study of a FFQ designed to measure *n-3* LC-PUFA[552] and none is available that has been assessed objectively against a biomarker. As discussed in **Section 6.1** a major aim of this study is to develop and validate a new FFQ (FishFQ).

6.3.1 Design of FishFQ

A prototype FishFQ was designed which focused on foods with the highest concentration of *n-3* LC-PUFA **(Appendix 3-3)**. The questionnaire was adapted from the EPIC FFQ, described in **Section 6.2.1**, which has been previously validated for use in a population of similar age. The EPIC FFQ combines fish and fish based foods in 6 line entries: fried fish, fish and fish cakes, white fish, oily fish, shellfish and fish roe. The FishFQ allows separation of these foods and also includes additional foods that contain significant amounts of *n-3* LC-PUFA. A food is considered a "good source" of a specific nutrient if it contains 10% more of the daily value for a given nutrient than the comparison food.[605] However, there are no specific DRVs for DHA or EPA and therefore good sources cannot be described in this way. For the purposes of this study, we included sources of *n-3* LC-PUFA identified through the most recent National Diet and Nutrition Survey (NDNS) available at the time of study.[606, 606606, 606606, 606606, 606606, 606606, 606606, 606606, 606606, 606606, 606606, 606606, 606606, 606605, 605606] Twenty-seven items were included

in the original questionnaire which included seven frequency categories ranging from never to more than twice daily.

6.3.2 Evaluation of FishFQ

The FishFQ was given to a sub-set of 20 participants who had agreed to take part in the DHA supplementation RCT. After completing the questionnaire, participants were asked to complete an evaluation form designed to assess understanding and ease of use of the FishFQ. The evaluation form comprised seven questions that addressed clarity, length and content, and asked whether participants could complete the questionnaire unaided. One further question sought the opinion of participants on the usefulness of assistance given by researchers. The evaluation form can be found in **Appendix 3-4** and results are presented in **Table 6-1**.

Almost all participants (18/20, 90%) asked to evaluate the FishFQ thought the content was clear, and (19/20, 95%) found it easy to understand. All indicated that length was appropriate and only two people need further explanation and assistance. Just over half said all foods that participants expected to be on the questionnaire were included and 16 out of 20 said foods they usually ate which they thought were sources of *n-3* were not included. A summary of foods expected to be included or eaten but excluded are listed in **Box 6** below. All participants indicated that explanations and help given by the research team were adequate.

Box 6 Foods expected to be included or omitted from FishFQ

Expected but omitted	**Eaten but not included**
Sunflower seeds/oil	Omega 3 spreads
Canned tuna	Omega 3 milk
Canned Herring	Omega 3 yoghurt
Canned kipper	Omega 3 eggs
Canned Mackerel	
Skippers (Brisling)	

Following evaluation, the questionnaire was amended to include foods reported to be regularly eaten and that were sources of *n-3* LC-PUFA. This included foods

fortified with *n-3* fatty acids, that were not included in the EPIC FFQ, and were not available when data collection for the NDNS (2003) took place **(Appendix 3-5)**. All oily fish were presented as either fresh or canned as some participants reported consuming both types. Skippers (Brisling or Spratts), a small herring, were included because they were reported to be consumed by three participants during the evaluation. Supermarket reconnaissance and discussion with manufacturers confirmed these as a popular product among consumers. Portion size estimates were obtained by administering the FishFQ in conjunction with a portion size booklet. The booklet was designed as part of this study and included colour photographs of foods included in the FishFQ **(Appendix 3-6)**. Foods were photographed on standard dinner plates and a range of portion sizes were included corresponding to small, medium and large portions as described.[604]

The amended questionnaire was designed in teleform (Cardiff software) scanned data entry system to facilitate ease of data entry. The new FFQ included 31 items and a column where portion size could be clearly indicated. Nine frequency choices were given for each food: never; one to two per month; one per week; two to four per week; five to six per week, one per day two to three per day, four to five per day, five to six per day. The higher frequencies were added to accommodate newly emerging omega 3 enriched foods, for example milk, that may have been consumed more frequently.

6.3.3 Administration of FFQ

The FishFQ was administered prior to and following supplementation. Participants were asked to record how often on average they consumed each item during the past year. The FFQ was adapted from the EPIC FFQ and allowed separation of *n-3* foods which were previously grouped together and thereby aimed to improve estimates of intake. As for the EPIC FFQ, the questionnaires were completed whilst the subject attended the research centre and assistance was given, when requested, from myself or other trained researchers (dietitians and nutritionists) who checked for completeness of entry after completion.

6.3.4 Analysis Methods of FFQ

The FishFQ was analysed in accordance with the dietary analysis manual described in **Appendix 3-2, Section 6.3.2.** Two spreadsheets were designed in Microsoft excel to provide templates for conversion of frequency data to amount in grams of daily food consumption. Frequencies were multiplied either by amounts in grams according to standard portion sizes[604] or according to portion sizes depicted in the omega-3 portion size booklet **(Appendix 3-6).**

6.3.5 Interim analyses

6.3.5.1 Agreement between dietary assessment methods

After administration of the FishFQ and the EPIC FFQ to the first 100 study participants agreement between the two questionnaires was assessed using the Bland and Altman (1986) technique.[607] This method of analysis assesses agreement of methods in groups, which is defined as the bias, and also assesses agreement in individuals, defined as the limits of agreement (± 2 s.d. of the bias). The relative bias was calculated as the mean difference in DHA and EPA intake between the two FFQs. Results of the Bland and Altman analysis are reported in **Chapter 10, Section 10.3.1**.

6.3.5.2 Agreement between portion size estimates

Agreement between standard and reported portion size estimates was also assessed using the Bland and Altman technique. Results are reported in **Chapter 10, Section 10.3.2**.

6.4 24-Hour Recall Method

6.4.1 Introduction

The 24-hour recall method was used to assess dietary intake in a sub-group of participants. This method was included to provide a reference dietary assessment method for validation of the newly developed FishFQ. Validity was assessed using the method of triads – an estimating technique used in path analysis **(Section 6.5.4.1)**.

6.4.2 Design of 24 hour recall method

The aim was to collect at least five days of 24-hour dietary recalls from willing participants. During visit 1, the procedure for collection of 24-hour dietary recalls was explained and only participants available and amenable to researchers contacting them during evenings and weekends to conduct telephone interviews were included. A dietary recall form was designed for the study based on a previous form used in the INTERnational Study of Macronutrients, Other Factors and Blood Pressure (INTERMAP) study. The form was designed to capture information relating to 1) meal type, e.g. whether home prepared or eaten out, 2) foods and drinks consumed during eating episode, 3) preparation methods, e.g. use of fat and salt and 4) cooking methods. A column for the addition of a food code was included and two extra columns allowed entry of either a standard portion size (wt 1) or portion size estimated from selected food photographs from the MAFF Photographic Atlas (wt 2).

6.4.3 Administration of 24-hour Dietary Recall

Twenty four hour recalls were collected during telephone interviews conducted by research assistants who were qualified dietitians, nutritionists or dietetic assistants as described previously.[480-482] Morgan and colleagues (1987) compared the use of telephone with personal interview dietary assessments and concluded that the telephone interview provided comparable data with less effort and cost. Response rates were 71-81 percent for telephone interviews compared with 72–83 percent for personal interviews.[483]

Research assistants were trained by myself, a registered dietitan, who was experienced in collection of dietary data in both clinical and research settings. Participants were allocated sequentially to a research assistant who then attempted to collect 7 recalls from each subject. Data collectors were asked to obtain records representing each day of the week if possible. A summary sheet was provided where research assistants could record progress in obtaining records and monitor collection attempts.

6.4.4 Analysis of 24-hour dietary recalls

Analysis of 24-hour recall was carried out by nutritionists and dietitians based at the Strangeways Research Laboratory, University of Cambridge. This centre was chosen because it uses a purpose built programme for analysis of 24 hour recalls – Data Into Nutrients for Epidemiological Research (DINER).[608] This method uses data from McCance and Widdowson's composition of foods complemented with additional data from reliable sources. For example, only about 30% of foods in McCance and Widdowson's Composition of Foods have been analysed for fatty acid content. When a food described on a dietary recall form is entered and information on fatty acid content is unavailable, DINER uses a mapping procedure to identify similar foods that do contain this information.

6.5 Validation of Dietary Assessment Methods

To assess compliance with the RCT protocol, we examined how supplementation with DHA was reflected in the concentration of fatty acids in erythrocyte (red blood cell [RBC]) membranes. To assess validity of dietary assessment methods used in the study, we evaluated relationships between dietary fatty acids measured against the EPIC and Fish FQs with multiple 24-hour dietary recalls and biomarkers of fatty acid status.

6.5.1 Biomarkers of fatty acid status

Biomarkers of fatty acid used in the present study included the *n-3* LC-PUFAs DHA and EPA both separately and combined to provide an omega-3 index. The omega-3 index is defined as EPA+DHA as a percentage of total RBC fatty acids and has been proposed as a risk factor for CHD.[609] The scientific basis for this comes from two major studies where RBC EPA+DHA concentrations were correlated with primary risk of cardiac arrest.[360, 362] In both studies the risk of sudden cardiac death was reduced by 90% in subjects with the highest compared with the lowest *n-3* concentrations. Harris and colleagues further propose cut-points can be used to identify individuals at low, intermediate and high risk based on their omega-3 status.[609] Dose response studies were used to explore effects of supplementation with EPA+DHA on RBC concentration. Doses of 500 and 1000 mg taken for 5 months achieved *n-3* indices of 8 and 10% respectively.

Applying these levels to results from previous studies it was estimated that a cardioprotective effect was present at an index of 8% and the greatest risk of CHD death was associated with an index below 4%. Intermediate risk fell between these two points.[610]

6.5.2 Comparisons of EPIC FFQ with 24-hour recall and biomarker

A total of 324 subjects consented to participate in the RCT. All subjects agreed to complete the EPIC FFQ at baseline. Questionnaires were assessed for completeness and only one subject was excluded on the basis of an incomplete EPIC questionnaire.

6.5.3 Comparisons of EPIC FFQ with biomarkers of fatty acid intake.

RBC fatty acid concentration was measured in 268 participants. Two hundred and thirty two subjects completed the FFQ at baseline (visit 1) and post intervention (visit 2). Please see **Chapter, 10, Figure 10-4** for flow of subjects through the dietary assessment study. There were no reports of supplements containing fish or fish oils being used, therefore fatty acid intakes represent intake from food alone. Intakes of fatty acids assessed with the EPIC FFQ were adjusted for energy intake in regression analyses.

6.5.4 Validation of FishFQ

Validation of the FishFQ was achieved by comparing intake of DHA, EPA and combined DHA+EPA (omega-3 index) as estimated by 5 days of dietary recall and biomarkers (fatty acids as percentage total red blood cell membrane (RBC) fatty acids). Therefore, only subjects who completed all these dietary assessments were included in the validation study. Eight subjects were excluded on the basis of inadequate completion of the FishFQ. Therefore 78 subjects were included in the FishFQ validation study.

6.5.4.1 The Method of Triads

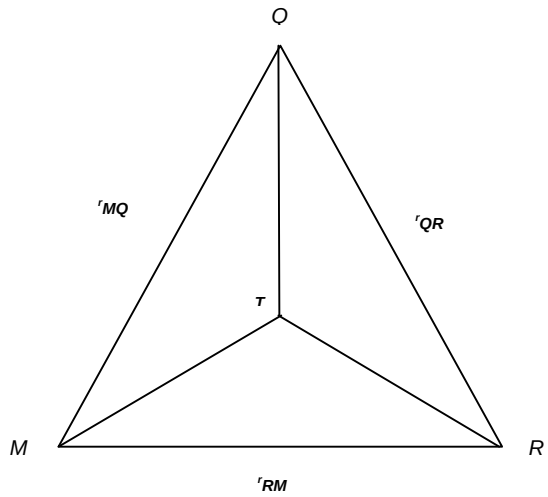
Studies assessing the relative validity of dietary questionnaires usually include a reference dietary assessment method such as weighed records or multiple 24-hour recalls of dietary intake. The correlation between measurements obtained

using questionnaires (*Q*) and the subject's true long-term intake (*T*) of foods or nutrients can be estimated from the correlation between the two dietary assessment instruments to provide a validity coefficient (p_{QT}). As discussed in **Chapter 4, Section 4.5.3**, however, random errors may introduce bias because the same factors that affect the reference method may also affect the new method under test.

Validation studies assume random errors between dietary assessment methods are uncorrelated although in practice this is rarely the case, and inter method correlations may be over-estimated.[611] The use of biochemical markers in dietary assessment studies reduces the risk of bias because random errors are likely to be independent of those attributed to questionnaires and food records. The problem of correlated errors can therefore be overcome with the use of triangular comparisons. The present analysis applies the method of triads to assess agreement between the new method (*Q*) (FishFQ), the reference dietary assessment method (*R*) (24-hour recall) and the biochemical marker (*M*) (RBC fatty acid concentration).

Triangular comparisons make the assumption that random errors are independent but that each measurement has a linear relationship with the truth. Structural equations can be applied to evaluate the contribution of systematic scaling biases as described by Loehlin (2009).[612] For investigations where estimates of correlations between questionnaire measurements and true intake values are required the method of triads can be applied **(Figure 6-2)**.

Figure 6-2: Triangular comparisons between the questionnaire (*Q*), reference method (*R*) and biochemical marker (*M*) using the method of triads.



$^{r}QR = {}^{P}QT \times {}^{P}RT$

$^{r}RM = {}^{P}RT \times {}^{P}MT$

$^{r}MQ = {}^{P}QT \times {}^{P}MT$

$^{P}QT = \sqrt{}\,{}^{r}QR \times {}^{r}MQ/{}^{r}RM$

$^{P}RT = \sqrt{}\,{}^{r}QR \times {}^{r}RM/{}^{r}QM$

$^{P}MT = \sqrt{}\,{}^{r}MQ \times {}^{r}RM/{}^{r}QR$

The 95% CIs for the validity coefficients were estimated using bootstrap sampling. The bootstrap method uses a re-sampling technique where up to 1000 samples of equal size (n = 78) are obtained by replacing values drawn from the original population. This allows estimation of the sampling error in the original population and evaluation of the confidence intervals.

6.5.4.2 Sample Size Calculation

The minimum projected sample size was 125 subjects. This estimate was based on an expected correlation of 0.5–0.6 between dietary *n-3* dietary intake assessment methods and biochemical markers of *n-3* intake reported in previous studies [546, 552] and was sufficient to detect a 0.25 standard deviation difference between dietary recall and FFQ at 5% significance and with 95% power. Professor T.J. Cole at the Department of Paediatric Epidemiology and Biostatistics, University College London, Institute of Child Health, provided statistical advice for this sample calculation.

6.5.5 Correlations between dietary assessment methods

All dietary and RBC data were assessed for normality using the Kolmogorov-Smirnoff test. Where significant skewness or kurtosis was identified data were transformed to improve normality. In all cases natural log transformation improved data distribution.

Pearson's correlation coefficients were calculated between the two dietary assessment methods and between each dietary assessment and the biomarker (i.e. FishFQ and 24-hour recall; FishFQ and biomarker; 24-hour recall and biomarker). Correlations were evaluated as poor ($r < 0.2$), moderate ($r = 0.2–0.6$) or good ($r > 0.6$).[555]

6.5.6 Classification into thirds of the population distribution

Agreement on category level between *n-3* fatty acid intake estimated using the EPIC and FishFQ and RBC concentrations was examined by classification into appropriate portions of the distribution of intake and membrane concentration respectively. The proportions of individuals classified in the same third of the distribution (exact agreement) and into extreme thirds of the distribution (gross misclassification) were calculated using descriptive statistical procedures to identify cut points for equal thirds of the distribution. The crosstabs facility was used to tabulate results.

6.5.7 Assessment of Reliability

Reliability of the EPIC and FishFQ was tested by administering the same dietary questionnaires at baseline and 4 months later. Pearson's correlation coefficients were calculated to assess reproducibility.

6.5.8 Regression Analyses

General linear modelling was used to investigate relationships of dietary patterns and *n*-3 fatty acids with measures of atherosclerosis and CVD risk factors. DHA and EPA status was assessed through measurement of red cell membrane fatty acid concentrations. Dietary patterns were derived using principal component analysis (PCA) as described below.

6.5.9 Principal Component Analysis

Principal component or factor analysis is a mathematical technique, introduced by Spearman (1904), which can be applied to reduce a complex dataset of many components to a smaller set with fewer variables. PCA identifies linear combinations of the original observed variables on the basis of a correlation

matrix that accounts for most of the total variance in the data. Coefficients of variation which define the linear combinations are termed 'factor loadings' and represent the correlations of the variables in the original dataset. A large factor loading indicates a variable is strongly associated with its component. The first principal component extracted accounts for most of the variance in the sample. Successive components explain the remaining variance and each component is independent of others.

Extracting the optimum number of components is a challenge and several selection methods have been proposed. The Cattell (1966)[613] scree test and the Kaiser (1960)[614] rule are the most often used procedures to determine the number of components. They are both based on inspection of the correlation matrix eigenvalues which are calculated as the sum of each squared factor loading for a given component. Cattell's recommendation is to retain only those components above the point of inflection on a plot of eigenvalues ordered by diminishing size. This allows a cut point to be set for selection of components based on a visual trailing of factors on the plot **(Figure 6-3)**.

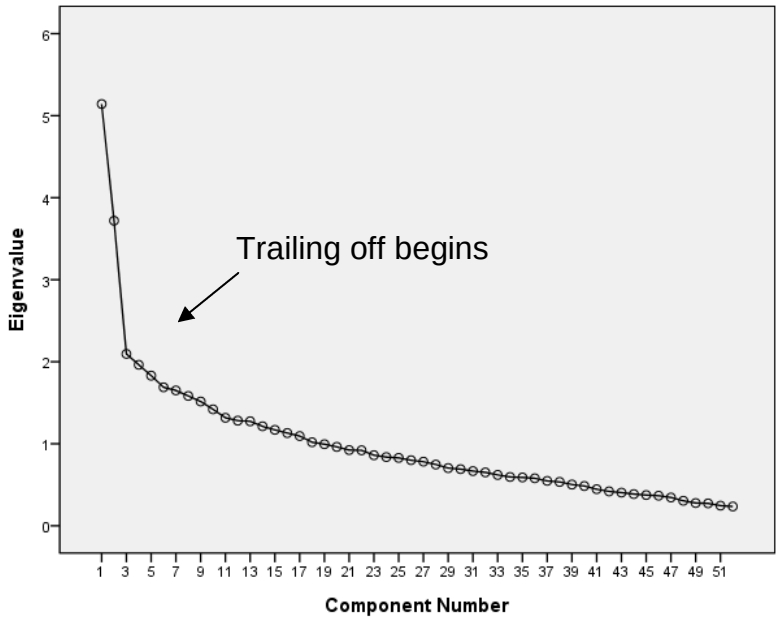
Dietary data were collected using the EPIC FFQ as described in **Section 6.2.1.1.** For deriving dietary patterns food items were re-classified into food groups based on similar nutrient content and common usage. 52 food groups were identified for further analysis. The sum of foods and beverages within each of the 52 food groups was calculated to provide a total daily frequency of intake for foods or beverages assigned to each food group.

Several food and beverage items were not categorized into food groups as they were thought to reflect specific dietary behaviours (e.g. coleslaw, juice, tea, liver, liver sausage, chicken or other poultry, fish roe). They were left as separate items. Principal components analysis was conducted to identify the main dietary patterns from the included food items.

All food groups entered in the PCA were expressed as the sum of daily frequency of use. The first PCA on the 52 food groups was performed to produce a scree

plot of eigenvalues of the derived factors as described earlier. Three factors were retained for further analysis based on the scree plot.

Figure 6-3: Scree plot for Principal Components Analysis of dietary data collected at baseline using the EPIC FFQ



A second PCA was conducted on these three components to derive factor loadings that would allow interpretation of dietary patterns. The size of the factor loading for a food group within a component characterises its contribution to the dietary pattern i.e. a larger food loading identifies a greater contribution of a particular food group to a specific factor. To improve interpretability and minimise the correlation between the factors, factors were rotated with an orthogonal (varimax) rotation.

The first dietary pattern was characterised by high daily intakes of all kinds of vegetables, fresh fruits, other fruits, oily fish, nuts, vegetarian foods, low fat dairy products and cereals. The second factor was characterised by processed meats, high fat dairy products, non oily fish products, savoury pies, combination meals, red meats and poultry and the third factor was characterised by confectionary, salty snacks, convenient fish products, biscuits and cakes, potato products and high energy drinks **(Table 6-2)**. A merged table consisting of the food group frequencies, factor scores was constructed and quintiles according to dietary

pattern scores were calculated. Cut points for 5 equal groups were calculated using the frequencies command in PASW statistics, version 18 and new variables were created using the transform command.

6.5.10 Assessment of normality

Distributions of fasting VLDL, triglyceride, insulin, CRP and cotinine concentrations and sum of skinfold thickness were right skewed and so were $\log_e$ transformed and then multiplied by 100 prior to analysis.[601] The standard deviation for 100 $\log_e$ transformed data represents the coefficient of variation of the original data, while regression coefficients represent the percentage difference in outcome per unit change for the independent variable.[601]

6.5.11 General Linear Modelling

Linear regression was used to calculate unadjusted means of participant characteristics, measures of vascular structure and function and CVD risk factors in each quintile of dietary pattern score. In addition to the unadjusted analysis, multivariate linear models were used to assess relationships between outcomes and quintiles of dietary pattern score. Model 2, adjusted for age and sex, social and lifestyle factors, total energy intake and CVD risk factors known to affect outcomes including blood pressure, fasting lipid and insulin concentrations and measures of obesity. Results of analyses of relationships between dietary patterns and CVD risk factors are reported in **Chapter 8**.

Effects of dietary fats on health have been the subject of much diet-disease research. In particular, as discussed in **Chapter 3**, health benefits associated with long-chain *n-3* LC-PUFA consumption have been widely reported.[330] Although a large body of observational and experimental evidence supports the hypothesis that these fatty acids are protective against secondary prevention of CHD **(Chapter 3, Section 3.5.1)**, and observational evidence further suggests benefits for primary prevention of CVD **(Chapter 3, Section 3.3)**, no experimental studies have been conducted to investigate the hypothesis that *n-3* LC-PUFA protects against the development of atherosclerosis in healthy young adults.

The present RCT was designed specifically to investigate the hypothesis that supplementation with DHA benefits vascular function and is therefore protective against development of primary CVD. Biochemical markers of fatty acid status obtained as part of the RCT allowed investigations into associations of fatty acid status with vascular health and CVD risk factors. Data obtained from red cell membrane fatty acid analysis, as described in **Chapter 5, Section 5.2.6.3** were re-coded into quintiles of fatty acids expressed as a percentage of total red cell membrane fatty acids **(Chapter 5, Section 5.2.6.3)**.

The development of validated dietary assessment methods within the RCT also allowed investigation of dietary exposures and CVD risk factors. Data were sorted to quintiles of fatty acid intake according to dietary assessment using the EPIC FFQ at baseline. The EPIC questionnaire was chosen in preference to the new questionnaire as this provided the greatest subject number. Multivariate linear regression was used to investigate relationships between *n-3* LC-PUFA, vascular structure and function and CVD risk factors. The same 2 multivariate linear models were used to assess relationships between these outcomes and quintiles of *n-3* LC-PUFA intake according to dietary assessment and biomarker status. Results of these analyses are reported in **Chapter 9**.

Table 6-1 Evaluation of FishFQ

	Strongly Agree	**Agree**	**Disagree**	***P***
Content clearly explained	**12**	**6**	**2**	
Easy to understand	14	5	1	
Acceptable Length	10	10	0	
Assistance required	2	0	18	
Foods expected present	3	8	9	
Foods expected missing	3	1	16	
Explanations and help from researchers				
	Excellent	**Good**	**Fair**	**Poor**
	5	13	2	0

Table 6-2 Factor loading of various food items in the three principal components identified (loadings above 0.25 are shown in bold)

	Dietary Patterns		
Food Item	**Health Conscious**	**Meat Based**	**Snack**
Green Leafy Vegetables	**0.686**	0.007	0.004
Yellow Vegetables	**0.569**	-0.147	-0.040
Cruciferous vegetables	**0.516**	0.058	0.116
Alium Vegetables	**0.388**	0.148	0.244
Beans and Legumes	**0.473**	0.107	0.120
Other vegetables	**0.645**	0.035	0.160
Fresh Fruits	**0.590**	-0.084	0.077
Citrus Fruits	**0.394**	-0.067	0.038
Other Fruits	**0.486**	0.017	0.001
Tomatoes	**0.401**	**0.471**	0.033
Oily fish	**0.465**	-0.101	0.055
Non-oily fish and seafood	**0.391**	-0.091	0.328
Non-oily fish products	0.172	**0.509**	0.002
Convenient Fish Products	-0.040	0.065	**0.533**
Red Meats	0.093	**0.446**	**0.309**
Processed meats	-0.124	**0.666**	0.089
Organ meats	0.001	**0.309**	-0.065
Poultry	0.143	**0.434**	0.158
Eggs and egg products	0.170	0.172	-0.021
High-fat dairy products	0.047	**0.587**	-0.070
Low-fat dairy products	**0.256**	0.205	0.132
Nuts	**0.318**	-0.075	0.206
Vegetarian foods	**0.300**	-0.223	-0.162
Cereals	**0.240**	-0.028	-0.049
Whoegrain/meal products	**0.322**	**0.338**	0.014
Refined Grain Products	-0.259	**0.332**	**0.356**
Potato Products	0.073	**0.248**	**0.477**
Combination meals	-0.029	**0.456**	**0.376**
Savoury Pies	-0.121	**0.468**	.143
Soups	**0.278**	0.235	0.085
Saturated fats	-0.044	**0.311**	0.003
Polyunsaturated fat	-0.070	0.170	0.108
Spreads (regular)	-0.106	0.182	0.204
Spreads (low fat)	0.028	0.019	0.195
Low-fat dressings/sauces	**0.388**	0.186	0.069
High fat dressings/sauces	0.104	**0.409**	0.135
Coleslaw	0.152	-0.012	**0.353**
Salty snacks	-0.161	0.255	**0.581**
Confectionary	0.041	-0.064	**0.650**
Biscuits and cakes	0.029	0.100	**0.493**
Dairy Dessert	0.098	0.231	**0.347**
Sugars and Preserves	-0.175	0.178	**0.264**
High Energy Drinks	-0.002	0.096	**0.402**
Low Energy Drinks	0.156	-0.168	**0.288**
Condiments	0.136	-0.043	0.241
Juice	0.172	0.227	0.167
Tea	-0.019	0.216	-0.060
Coffee	0.058	-0.099	**0.314**
Chocolate Drinks	0.146	-0.047	**0.306**
Beer	-0.071	0.213	-0.062
Wine	0.209	0.021	0.023
High alcohol beverages	0.102	-0.039	0.149

Chapter 7

Docosahexaenoic Acid Supplementation, Vascular Function and Risk Factors for Cardiovascular Disease; a Randomised Controlled Trial in Young Adults

Our body is a machine for living. It is organized for that, it is its nature. Let life go on in it unhindered and let it defend itself, it will do more than if you paralyze it by encumbering it with remedies.

Leo Tolstoy

Section 3: Results

7.1 Introduction

Increased fish consumption has been shown to reduce the risk of CVD in both epidemiological studies[275, 327, 328] and RCTs[329-331] **(Chapter 3, Section 3.1)**. In **Chapter 2, Section 2.5.2.2** a comprehensive review found that beneficial effects on vascular function are associated with dietary LC-PUFAs, in particular with EPA (20:5*n-3*) and DHA (22:6*n-3*). It is well established that high intakes of *n-3* LC-PUFA have antithrombotic effects that operate through the production of less pro-inflammatory eicosanoids.[382] However, such effects require high doses (~15g/d) that are considered pharmacological and outside of the normal dietary range (1-2g/d).

Alternative mechanisms that involve *n-3*-LC-PUFA include effects on endothelial function. Metabolites derived from EPA and DHA may have protective roles during endothelial activation but exact mechanisms remain elusive. In particular DHA has been associated with improved endothelial function. In a previous study we reported associations between greater erythrocyte membrane DHA concentrations and endothelial function assessed using the FMD technique. In smokers, *n-3* fatty acids were positively related to flow-mediated dilatation (1% increase in plasma DHA was associated with 0.045 mm (0.5 SD), 95% CI: 0.011, 0.079, increase in FMD [*P* = 0.01]).[568]

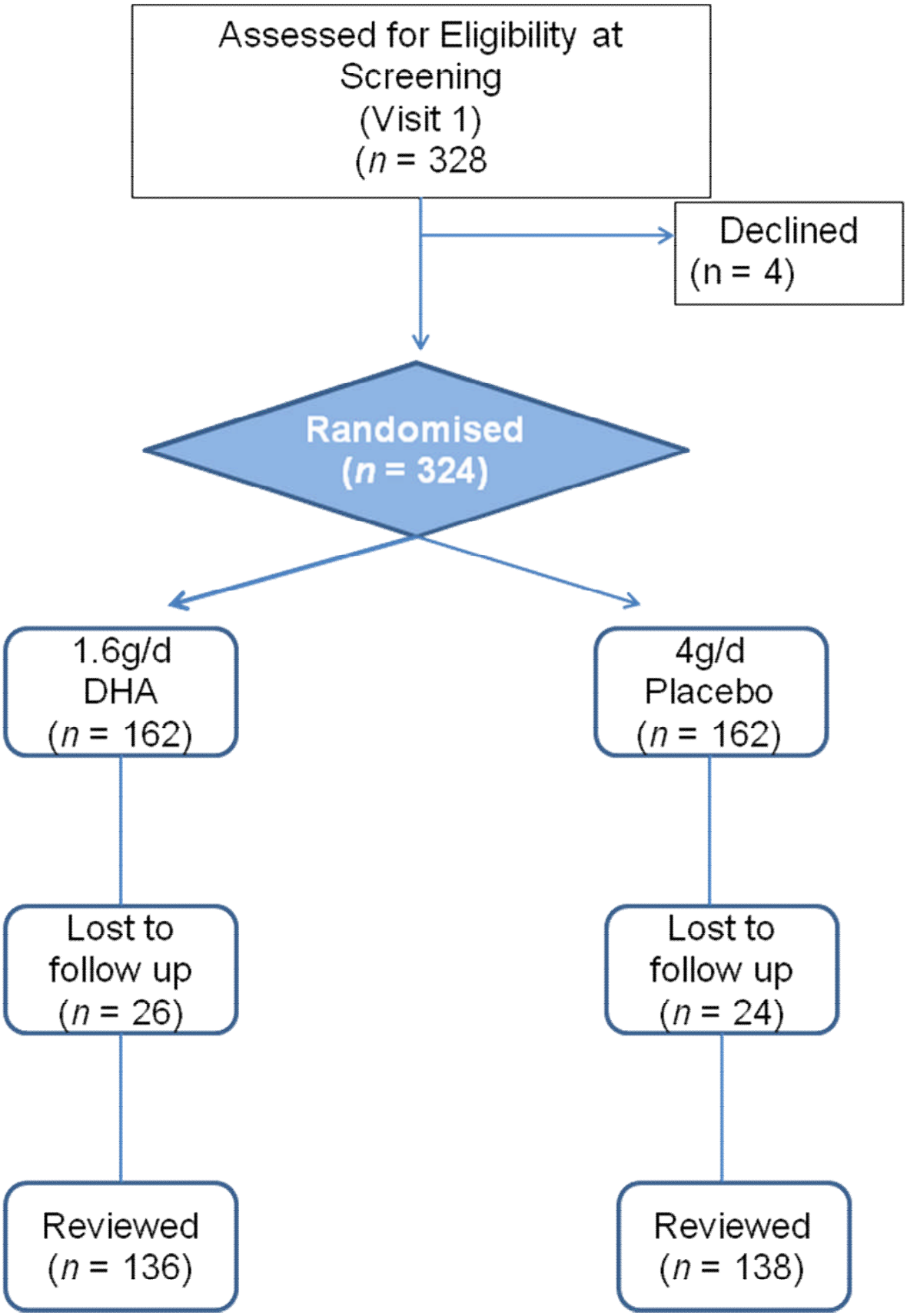
To our knowledge, the current study is the first RCT to assess effects of DHA supplementation on endothelial function in healthy young adults.

7.2 Methods overview

The hypothesis that supplementation with DHA improves endothelial function and CVD risk factors was tested in healthy volunteers (n=324) aged 18-37 years. Participants were randomly assigned to receive either supplements providing 1.6g/d DHA suspended in 2.4g olive oil or placebo containing 4g olive oil/day. Both oils were contained in gelatinized capsules and given as 8 x 500mg capsules for 16 weeks **(Figure 7-1)**.

274 subjects completed the intervention and FMD was measured in the brachial artery before and after supplementation in 268 subjects **(Figure 7-1)**. Methods are reported in detail in **Chapter 5**.

Figure 7-1 Flow of Participants Through Randomised Controlled Trial



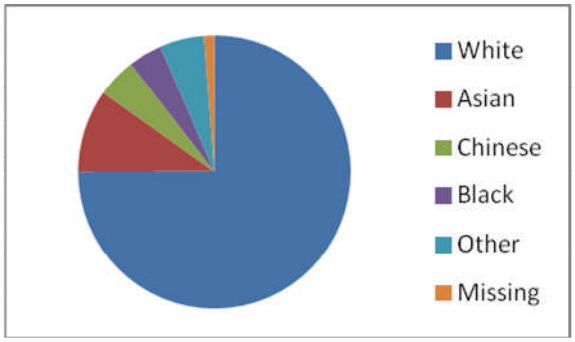
7.3 Results

The characteristics of the study population are summarized in **Table 7-1**. CVD risk factors in this young population compared favourably with those reported for the general adult population in the UK.[615] For example, mean BMI for men in the n-3 in Healthy Young People (N3HYP) study was 24 compared with 27 and for women was 23 compared with 28 in the Health Survey for England.[615]

7.3.1 Baseline characteristics of study participants

Most (242/324, 75%) participants were of white Caucasian origin **(Figure 7-2)**. About one third (113/324; 35%) were currently students and most participants (57%) were in non-manual occupations **(Table 7-1)**.

Figure 7-2 Ethnicity of study participants



Both intervention groups were closely matched for demographic, anthropometric and socio-economic variables, biochemical and cardiovascular risk facts and vascular measures at the baseline (randomisation) visit **(Tables 7-1 to 7-3)**.

7.3.2 Compliance and Monitoring

Subjects were sent weekly text messages to remind and encourage consumption of dietary supplements. Monitoring was carried out throughout the intervention period by weekly telephone calls where information on compliance and adverse events was collected. Data were available from 274 subjects (138 control; 136 test). There were no serious adverse events in either group and the supplements were well tolerated. The most commonly reported side effects of

supplementation were mild gastrointestinal discomfort. These are summarized in **Table 7-4**.

Compliance with dietary supplements was assessed by counting the number of capsules returned at the end of the intervention period and by measurement of DHA status expressed as percentage erythrocyte total fatty acids (RBC%FA). DHA supplemented individuals had higher red cell DHA concentration post-intervention compared with controls **(Table 7-5)**. DHA supplemented subjects had a significant increase in RBC omega-3 index (DHA+EPA RBC%FA) following supplementation (mean pre-intervention 6.5%, post-intervention 10.2%, $P < 0.0001$). There was no increase in omega-3 index in control subjects following intervention (6.2% pre and post intervention, $P = 1$). A greater proportion of DHA supplemented individuals had an omega 3 index greater than 8% following supplementation compared with baseline (76%, $n = 100/131$, 22% n = 35/160 respectively, $P < 0.0001$).

7.3.3 Primary Outcome

After adjustment for baseline diameter, absolute FMD of the brachial artery was significantly lower in the DHA supplemented group compared with controls (mean difference: -0.03 mm; 95% CI: -0.005 - -0.06 mm [$P = 0.02$]) **(Table 7-7)**. Adjustment for known confounders (age, sex, skin and room temperature, LDL and TG concentrations) did not attenuate the effect which remained significant. Other vascular outcomes did not differ between randomised groups.

7.3.4 Secondary Outcomes

There was no difference in blood pressure or fasting concentrations of insulin, glucose or CRP between randomised groups. However, fasting concentrations of VLDL and TG were lower in the DHA supplemented group compared with controls (mean difference for VLDL: -28%; 95% CI:-15, – -40%; [$P < 0.0001$]; mean difference for triglycerides: -28%; 95% CI: -15 - -40% [$P < 0.0001$]). Dietary characteristics, based on food intake data collected using the EPIC food frequency questionnaire, were similar to those of the UK adult population **(Table 7-6).** Fat intake was similar to that reported in the National Diet and

Nutrition Survey published just prior to commencement of the RCT.[606] For both men and women saturated fat intake was above UK COMA recommendations but slightly lower than in the NDNS.[159] Monounsaturated fatty acid intake was below the recommended intake.

Dietary intake of total fat and fatty acid sub-fractions amongst the study population, estimated from the EPIC FFQ were compared with COMA (1991,1994) recommendations.[159, 616] Current advice from the UK Scientific Advisory Committee for Nutrition is to consume two portions of fish per week, one of which should be oily. This would provide approximately 450mg/day *n-3* LC-PUFA. Total long chain *n-3* polyunsaturated fatty acids met the recommended amount. Intake of the *n-3* essential fatty acid alpha linolenic acid met the recommended intake for men and women.

7.3.5 Secondary analyses

The effect of DHA supplementation on FMD (expressed both as the absolute maximal change between pre- and post hyperaemic brachial artery diameter adjusted for pre-hyperemic diameter and maximal percentage change in diameter) was confined to men **(Table 7-7)**.(randomised dietary group x gender interaction: $P = 0.01$). Mean arterial blood pressure was lower in males in the intervention group following supplementation (randomised dietary group and gender interactions for mean SBP [$P = 0.04$]).

There were no significant differences in energy, macronutrient and fatty acid intakes in randomised groups at baseline and after completing the intervention **(Table 7-8)**.

Table 7-1 Subject Characteristics at Baseline

	All			***Males***			***Females***		
n	***324***			***120***			***204***		
Age, y	27.9	4.7	18.4 – 37.1	29	4.7	19-36.8	27.2	4.7	18.4 - 37
[2]Male gender, % ,	37	120	-	-	-	--	-	-	-
[2]Social class*:									
Manual, %, n	43	139	-	44	53	-	42	86	-
Non-manual, %, n	57	185	-	56	67	-	58	118	-
[2]Current smoker,%, n	13	43	-	12	14	-	16	32	-
Weight, kg	68.2	13.8	40.7 - 125.2	76	10.9	48.8 – 101.1	63.6	13.2	40.7 – 125.2
Height, cm	169.7	9.2	148.0 – 193.6	177.3	7.4	155.7 – 193.6	165.3	7	148 – 181.1
Body mass index, kg/m^2	23.6	3.9	15.5 – 44.4	24.1	3	17.7 – 31.1	23.3	4.4	15.5 – 44.3
% fat‡	24.0	5.6	10.6 – 39.1	23.4	5.2	13.9 – 35.1	24.3	5.7	10.6 - 39
[3]Sum skinfolds†, mm	44.7	41	16.0 – 127.7	40.4	41	18 - 103	52	38.6	16 - 128
Waist circumference, cm	77.3	10.1	58.2 – 130.8	83.4	8.1	62.3 – 101.9	73.7	9.5	58.2 – 130.8
Waist:Hip ratio	0.8	0.07	0.6 – 1.0	0.84	0.09	0.7 - 1	0.7	0.05	0.6 – 0.9
Blood pressure, mm Hg									
Systolic	112	11	78 – 157	117	10	78 -148.7	109	10	87 – 157
Diastolic	67	8	44 – 98	69	8	53 – 94.3	65	8	44 - 98
Mean Arterial	84	9	61 – 126	87	8	73 – 120.3	82	9	61 - 126
Pulse Pressure	45	6	20 – 67	48	6.2	20 - 67	44	6	29 - 61
Resting heart rate, beats/min	66	9	36 – 100	64	11	44 - 95	68	10	38 - 99

	All			*Males*			*Females*		
n	***324***			***120***			***204***		
Total cholesterol, mmol/L	4.2	0.8	1.0 – 7.6	4.3	0.8	2.4 – 7.6	4.2	0.8	1.0 – 6.8
LDL cholesterol, mmol/L	2.4	0.8	0.1 – 5.0	2.6	0.8	1.3 - 4.7	2.2	0.7	0.1 0 5.0
HDL cholesterol, mmol/L	1.4	0.3	0.7 – 2.7	1.3	0.3	0.7 – 2.3	1.5	0.3	0.7 – 2.7
[3]VLDL cholesterol	0.4	50	0.1 – 2.3	0.4	55	0.1 – 2.3	0.4	46.3	0.1 – 2.0
Total:HDL cholesterol ratio	3.1	1.0	0.7 – 6.8	3.5	1	1.8 – 6.4	2.9	0.9	0.7 – 6.8
LDL:HDL cholesterol ratio	1.8	0.8	0.1 – 5.1	2.1	0.8	0.8 – 4.7	1.6	0.8	1.6 - 0.8
[3]Triglyceride, mmol/L	0.9	50	0.3 – 5.0	0.9	55	0.3 – 5.1	0.8	46	0.3 – 4.4
Glucose, mmol/L	4.8	0.5	3.3 – 7.4	5	0.5	3.9 – 7.4	4.7	0.5	3.3 – 6.4
[3]Insulin, pmol/L	25.7	59	3.1 – 257.2	24	58	3.9 - 219	26.8	60	3 - 257
[3]Insulin resistance (HOMA)	0.8	63	0.1 – 10.3	0.7	63	0.1 - 10	0.8	64	0.08 – 10.3
[3]CRP, mg/L	1.0	121	0.1 – 27.4	0.8	114	0.1 - 15	1.1	124	0.1 – 27.4
[3]Cotinine, ng/ml	1.6	255	0.0 – 544	1.6	273	0.05 - 545	1.6	244	0.06 - 523

Data are mean, SD except: [1] range, [2]% (n) and [3]geometric mean (coefficient of variation). <5% loss of n for some variables. *According to the Registrar General's Classification (OPCS, 1995), †Measured at 4 sites: triceps, biceps, sub-scapular and supra-iliac.‡ Estimated from the sum of skinfold thickness using prediction equations of Durnin & Womersley (1974) and Siri (1961).

Table 7-2 Subject Characteristics at Baseline according to randomized dietary group

	Control						*DHA Supplemented*					
	All		**Male**		**Female**		**All**		**Male**		**Female**	
n	**162**						**162**					
Age, y	27.6	4.7	29.0	4.6	26.6	4.5	28.2	4.8	28.8	4.8	27.9	4.7
[2]Male gender, % , n			40	65	60	97			34	55	66	107
[2]Social class*:												
Manual, %, n	42	68	35	23	46	45	44	71	54	30	38	41
Non-manual, %, n	58	94	65	42	54	52	56	91	45	25	62	66
[2]Current smoker, n	14	23	12	8	15	15	13	20	9	5	14	15
Weight, kg	68.9	13.4	76.7	11.7	63.7	11.9	67.4	14.1	75.1	9.8	63.5	14.4
Height, cm	170.5	9.3	177.9	8.0	165.6	6.6	168.9	9.0	176.7	6.6	165.0	7.4
Body mass index, kg/m^2	23.6	3.5	24.2	2.8	23.2	3.9	23.6	4.3	24.1	3.2	23.3	4.8
% fat‡	23.9	5.6	23.3	5.4	24.3	5.8	24.0	5.5	23.6	5.1	24.3	5.7
[3]Sum skinfolds†, mm	48.4	39	42.3	39	53.0	36	46.4	43	38.7	43	51.0	41
Waist circumference, cm	77.5	9.7	83.6	8.0	73.4	8.5	77.1	10.6	83.0	8.4	74.0	10.3
Waist:Hip ratio	0.8	0.07	0.8	0.05	0.7	0.05	0.8	0.07	0.8	0.06	0.8	0.05
Blood pressure, mm Hg												
Systolic	113	11	118	10	109	10	111	11	114	8	109	11
Diastolic	67	8	69	8	65	7	67	8	68	7	66	8
Mean Arterial	84	9	88	9	81	8	83	8	86	6	82	9
Pulse Pressure	46	6	49	6	44	6	44	6	47	6	44	6

	Control						*DHA Supplemented*					
	All		**Male**		**Female**		**All**		**Male**		**Female**	
Resting heart rate, beats/min	66	9	65	9	67	8	66	10	63	8	67	10
Total cholesterol, mmol/L	4.2	0.9	4.3	0.9	4.1	0.9	4.3	0.8	4.4	0.8	4.3	0.8
LDL cholesterol, mmol/L	2.3	0.8	2.5	0.8	2.2	0.8	2.4	0.7	2.6	0.7	2.3	0.7
HDL cholesterol, mmol/L	1.4	0.3	1.3	0.3	1.5	0.3	1.5	0.4	1.3	0.3	1.5	0.4
[3]VLDL cholesterol	0.4	50	0.4	56	0.4	46	0.4	50	0.4	55	0.4	46
Total:HDL cholesterol ratio	3.1	1.0	3.6	1.0	2.8	0.9	3.1	1.0	3.5	1.0	2.9	0.9
LDL:HDL cholesterol ratio	1.8	0.8	2.1	0.9	1.5	0.7	1.8	0.8	2.1	0.7	1.6	0.8
[3]Triglyceride, mmol/L	0.8	50	0.9	55	0.8	46	0.9	50	0.9	55	0.8	46
Glucose, mmol/L	4.8	0.5	5.0	0.5	4.7	0.4	4.8	0.5	4.9	0.5	4.7	0.5
[3]Insulin, pmol/L	25.5	58	24.3	58	26.4	58	25.9	61	23.8	58	27.0	62
[3]Insulin resistance (HOMA)	0.8	61	0.7	63	0.8	61	0.9	66	0.7	64	0.8	67
[3]CRP, mg/L	0.9	120	0.8	108	1.0	128	1.0	122	0.8	126	1.2	120
[3]Cotinine, ng/ml	1.8	261	2.0	280	1.6	250	1.5	249	1.2	266	1.6	242

Data are mean, SD except: [1] range, [2]% (n) and [3]geometric mean (coefficient of variation). <5% loss of n for some variables. *According to the Registrar General's Classification (OPCS, 1995), †Measured at 4 sites: triceps, biceps, sub-scapular and supra-iliac.‡ Estimated from the sum of skinfold thickness using prediction equations of Durnin & Womersley (1974) and Siri (1961).

Table 7-3 Vascular Variables at Baseline

	All			*Control*						*DHA Supplemented*					
				All		**Male**		**Female**		**All**		**Male**		**Female**	
n	324			162		65		97		162		55		107	
Brachial Artery	mean	s.d.	range	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
Diameter, mm	3.2	0.6	1.9 – 4.9	3.2	0.6	3.7	0.4	2.8	0.3	3.2	0.6	3.8	0.4	2.9	0.4
Reactive hyperaemia, %	736	263	0 - 2289	713	249	713	232	778	240	759	276	665	216	808	292
Flow mediated dilatation, mm	0.27	0.1	0.06 – 0.6	0.27	0.1	0.27	0.1	0.27	0.1	0.27	0.1	0.26	0.1	0.28	0.1
Flow mediated dilatation, %	8.4	3.7	1.1 – 23.9	8.3	3.7	7.1	3.3	9.1	3.7	8.5	3.7	6.8	3.2	9.3	3.7
[1]Distension, mm	0.09	0.04	0.01 – 0.22	0.08	0.04	0.09	0.04	0.08	0.04	0.09	0.04	0.1	0.04	0.09	0.04
Distension, %	12.3	5.6	0.6 – 34.5	11.4	5.4	10.0	3.8	12.5	6.1	13.2	5.8	11.1	4.3	14.0	6.1
Distensibility coefficient [1]($x10^{-3}.kPa^{-1}$)	9.1	4.3	0.7 – 34.9	8.4	3.9	7.3	2.7	9.2	4.4	9.8	4.7	8.8	5.5	10.3	4.2
Right Common Carotid	6.5	0.5	5.0 – 8.0	6.5	0.5	6.8	0.5	6.3	0.5	6.5	0.5	6.8	0.5	6.3	0.4
Diameter, mm	0.7	0.2	0.2 – 1.4	0.7	0.1	0.7	0.2	0.7	0.1	0.7	0.2	0.7	0.2	0.7	0.1
Distension, %	11.3	2.6	2.5 – 20.4	11.6	2.4	11.0	2.5	12.0	2.4	11.1	2.7	11.1	3.4	11.2	2.4
Distensibility coefficient % ($x10^{-3}.kPa^{-1}$) [1]	34.2	9.0	8.1 – 119.1	33.8	7.3	30.3	6.2	36.3	7.0	34.5	10.3	33.0	14.4	35.3	7.5
Intima-media thickness, mm	0.55	0.08	0.4 – 1.0	0.54	0.08	0.56	0.1	0.53	0.07	0.54	0.07	0.56	0.08	0.54	0.07
Carotid-radial PWV, m/s	8.6	1.3	5.6 – 17.0	8.6	1.2	8.9	1.1	8.3	1.2	8.6	1.4	8.6	1.2	8.6	1.4

[1]n=110 in control and DHA supplemented groups. <5% loss of n for some variables

Table 7-4 Symptoms reported by participants during compliance monitoring

	Number of Participants Reporting Symptoms		
Symptom*	**All**	**Control**	**DHA Supplemented**
n	262	133	129
None, % (n)	85 (223)	86 (114)	84 (109)
Headache	7	3	4
Stomach Pain	8	3	5
Nausea	7	2	5
Bloating	13	6	7
Flatulence	21	12	9
Diarrhoea	8	4	4
Constipation	6	4	2
Itching	3	1	2
Skin rash/spots	4	2	2
Fatigue	4	1	3
Dizziness	3	2	1
Other Symptoms	8	3	5
Bleeding Gums	1	0	1
Cold/flu	2	1	1
Cough	1	0	1
Eyelids drooping	1	0	1
Thirsty	1	0	1
General Malaise	1	1	0
Menstrual discomfort	1	1	0

Table 7-5 Red Blood Cell Fatty Acid Concentrations Pre- and Post-Intervention

	Control						DHA Supplemented						Comparison of Randomized Groups (*P*)		
	All		Male		Female		All		Male		Female		All	Male	Female
n : Pre-intervention	156		65		91		160		54		106				
n: Post-intervention	135		56		79		131		50		81				
Docosahexaenoic, %															
Pre-intervention	5.3	1.7	5.2	1.8	5.3	1.6	5.5	1.7	5.3	1.8	5.6	1.6	0.3	0.8	0.3
Post-intervention	5.3	1.8	5.1	1.8	5.4	1.7	8.9	2.6	8.8	2.7	8.9	2.5	<0.0001	<0.0001	<0.0001
Eicosapentaenoic, %															
Pre-intervention	1.0	0.6	1.1	0.6	0.9	0.5	1.0	0.5	1.0	0.6	1.0	0.5	0.7	0.5	0.2
Post-intervention	0.9	0.5	1.0	0.5	0.9	0.5	1.3	0.6	1.2	0.6	1.4	0.6	<0.0001	0.01	<0.0001
***n-3* index** [1]															
Pre-intervention	6.2	2.1	6.2	2.3	6.3	2.0	6.5	2.1	6.3	2.3	6.6	2.0	0.3	0.9	0.2
Post-intervention	6.2	2.2	6.1	2.2	6.3	2.2	10.2	3.0	10.0	3.1	10.3	2.9	<0.0001	<0.0001	<0.0001
***n-3* index ≥8%, %, n**															
Pre-intervention	19, 30		17, 11		21, 19		22, 35		22, 12		22, 23		0.3	0.3	0.5
Post-intervention	16, 22		16, 9		16, 13		76, 100		70, 35		80, 65		<0.0001	<0.0001	<0.0001

[1] *n-3* index= Docosahexenoic **+** eicosapentenoic/ total fatty acid

Table 7-6 Dietary energy and fat intakes at baseline assessed using EPIC FFQ compared with UK National Diet and Nutrition Survey of adults aged 19-64 (Henderson, 2003) and COMA (1991) dietary reference values (DH, 1991)

Nutrient	*N-3 HYP Study*			*NDNS†*		*COMA*	
	All	***Males***	***Females***	***Males***	***Females***	***Males***	***Females***
n	*323*	*119*	*204*	*219*	*210*	*N/A*	*N/A*
Energy (Mj/d)	9.4 (3.8-16.8)	10.3 (4.5-16.8)	8.9 (3.8-14.4)	8.9 (5-14.9)	6.3 (3.3-9.6)	10.6	8.1
Fat (% Energy)	34.4 (19.7-34.4)	34 (19.7-49.6)	34.6 (20-51)	35.8 (26-47.2)	35.4 (22.3-46.1)	≤33	≤33
Saturated Fatty Acids (% energy)	11.8 (5.1-25.8)	11.7 (5.1-24.8)	11.8 (5.5-25.8)	13.2 (8.5-18.3)	13.2 (7.5-18.6)		
MUFA (% Energy)	11.3 (5.2-20.1)	11.1 (5.9-17.7)	11.4 (5.2-20.1)	12.3 (8.7-16.7)	11.7 (7.5-16.9)		
ALA (% Energy)	0.2 (0.04-0.8)	0.2 (0.04-0.6)	0.2 (0.05-0.8)			0.2	0.2
Cis *n-3* PUFA (% energy)	0.8 (0.2-1.4)-	0.8 (0.2-1.4)	0.8 (0.5-1.1)	1.0 (0.5-2.3)	1.0 (0.5-1.9)		
***n-3* LC-PUFA (% Energy)**	0.2 (0-1.1)	0.2 (0.01-0.71)	0.2 (0-1.1)	-	-	0.2g *n-3*-LC-PUFA/d	
DHA (% Energy)	0.09 (0-0.6)	0.07 (0-0.4)	0.09 (0-0.6)	-	-		
EPA (% Energy)	0.1 (0-0.4)	0.09 (0-0.3)	0.1 (0-0.4)	-	-		

† Based on age group 25-34 years for comparison.

Table 7-7 Vascular Variables and Cardiovascular Risk Factors Post-Intervention

	Control						DHA Supplemented						Comparison of randomized groups(*P*)		
	All		***Male***		***Female***		***All***		***Male***		***Female***		***All***	***Male***	***Female***
Completed study, %, n	*85*	*138*	*88*	*57*	*84*	*81*	*84*	*136*	*91*	*50*	*80*	*86*	*0.4*	*0.4*	*0.4*
Brachial Artery															
Diameter, mm	3.2	0.5	3.7	0.3	2.9	0.3	3.2	0.6	3.8	0.4	2.9	0.3	0.9	0.3	0.3
Reactive hyperaemia, %	713	232	632	253	700	200	735	243	672	228	773	246	0.5	0.4	0.9
Flow mediated dilatation, mm	0.29	0.1	0.32	0.1	0.27	0.1	0.26	0.1	0.25	0.1	0.27	0.1	0.02	0.002	0.8
Flow mediated dilatation, %	8.6	3.5	7.9	3.6	9.1	3.3	8.0	4.0	6.3	3.1	9.0	4.2	0.2	0.01	0.9
Distension, mm^2	0.09	0.04	0.1	0.03	0.08	0.04	0.09	0.04	0.1	0.04	0.08	0.04	0.5	0.2	0.9
Distension, %	12.6	6.9	11.1	6.9	13.8	6.7	12.6	5.4	11.5	4.5	13.3	5.7	0.9	0.8	0.6
Distensibility coefficient ($x10^{-3}.kPa^{-1}$)	8.9	4.0	7.5	2.5	10.0	4.7	9.2	3.7	8.3	3.2	9.7	3.9	0.6	0.2	0.7
Right Common Carotid															
Diameter, mm	6.5	0.5	6.7	0.5	6.3	0.4	6.4	0.5	6.8	0.4	6.2	0.4	0.4	0.4	0.2
Distension, mm	0.7	0.1	0.7	0.2	0.7	0.1	0.7	0.2	0.8	0.2	0.7	0.1	0.4	0.5	0.07
Distension, %	11.4	2.4	11.0	2.8	11.7	2.1	11.2	2.5	11.2	3.0	11.3	2.2	0.6	0.7	0.2
Distensibility coefficient %	34.1	7.8	30.2	6.6	36.9	7.4	34.0	7.5	31.3	7.0	35.6	7.3	0.9	0.4	0.3
Intima-media	0.55	0.09	0.56	0.1	0.5	0.07	0.55	0.07	0.56	0.06	0.5	0.0	0.7	0.9	0.3

	Control						DHA Supplemented						Comparison of randomized groups(*P*)		
	All		***Male***		***Female***		***All***		***Male***		***Female***		***All***	***Male***	***Female***
Completed study, %, n	*85*	*138*	*88*	*57*	*84*	*81*	*84*	*136*	*91*	*50*	*80*	*86*	*0.4*	*0.4*	*0.4*
thickness, mm												7			
Carotid-radial PWV, m/s	8.6	1.3	9.0	1.3	8.3	1.2	8.5	1.5	8.8	1.3	8.4	1.6	0.8	0.3	0.6
Blood pressure, mm Hg															
Systolic	110	11	116	10	105	9	110	11	113	7	108	12	0.9	0.07	0.2
Diastolic	65	7	68	6	62	7	65	8	65	7	64	9	0.9	0.05	0.1
Mean Arterial	82	8	87	7	79	7	82	9	83	7	81	10	0.7	0.02	0.2
Pulse Pressure	45	7	49	6	43	6	45	6	48	5	43	6	0.5	0.3	0.7
Resting heart rate, beats/min	64	9	63	10	65	8	64	9	61	8	65	10	0.7	0.2	0.7
Total cholesterol, mmol/L	4.2	0.8	4.3	0.9	4.2	0.8	4.4	0.8	4.5	0.9	4.4	0.8	0.1	0.2	0.3
LDL cholesterol, mmol/L	2.4	0.8	2.6	0.8	2.3	0.7	2.6	0.8	2.8	0.8	2.5	0.7	0.06	0.2	0.1
HDL cholesterol, mmol/L	1.4	0.3	1.3	0.3	1.5	0.3	1.5	0.4	1.4	0.3	1.6	0.4	0.08	0.04	0.6
[3]VLDL cholesterol, mmol/L	0.4	49	0.4	50	0.4	50	0.3	54	0.3	54	0.3	54	<0.0001	0.01	0.001
Total:HDL cholesterol ratio	3.2	1.0	3.5	1.0	2.9	0.9	3.1	1.0	3.4	1.0	3.0	1.0	0.8	0.6	0.7
LDL:HDL cholesterol ratio	1.8	0.8	2.1	0.9	1.6	0.7	1.9	0.9	2.1	0.9	1.7	0.8	0.6	0.9	0.3
[3]Triglyceride, mmol/L	0.8	49	0.9	49	0.8	49	0.6	54	0.7	54	0.6	54	<0.0001	0.009	0.001
Glucose, mmol/L	4.8	0.5	4.9	0.4	4.6	0.5	4.7	0.6	4.9	0.7	4.6	0.5	0.7	0.7	0.9
[3]Insulin, mU/L	27.7	55	28.5	58	27	54	29.3	61	29.0	67	29.5	58	0.4	0.8	0.3
[3]Insulin resistance	0.8	58	0.8	58	0.8	59	0.9	68	0.9	74	0.8	64	0.4	0.8	0.4

	Control						DHA Supplemented						Comparison of randomized groups(*P*)		
	All		***Male***		***Female***		***All***		***Male***		***Female***		***All***	***Male***	***Female***
Completed study, %, n	*85*	*138*	*88*	*57*	*84*	*81*	*84*	*136*	*91*	*50*	*80*	*86*	*0.4*	*0.4*	*0.4*
(HOMA), µm/L															
[3]**CRP, mg/L**	1.0	114	1.0	110	1.1	118	1.0	123	0.6	111	1.2	123	0.7	0.06	0.6

Data are mean, SD except[1] % (n) and [3]geometric mean (coefficient of variation). <10% loss of n for some variables except [2] n=105

Table 7-8 Dietary intakes before and after treatment with *n-3* LC-PUFA by randomly assigned treatment groups

	Control						DHA Supplemented						Comparison of Randomized Groups (P)		
	All		***Male***		***Female***		***All***		***Male***		***Female***		***All***	***Male***	***Female***
n Pre-intervention	161		64		97		162		55		107				
n Post-intervention	137		56		81		136		50		86				
Energy (Mj/d)															
Pre-intervention	9.1	2.8	10.2	2.9	8.4	2.5	9.8	2.5	10.5	2.5	9.4	2.5	0.04	0.83	0.05
Post-intervention	8.7[3]	2.9	9.7	2.7	8.0	2.9	9.4[3]	2.6	9.8	2.4	9.1	2.7	0.87	0.12	0.1
Protein (% energy)															
Pre-intervention	16.1	3.5	16.6	3.5	15.8	3.4	15.8	2.6	16.6	2.6	15.4	2.5	0.67	0.88	0.6
Post-intervention	15.8	3.4	16.4	3.5	15.5	3.2	15.7	2.5	16.1	2.4	15.5	2.7	0.35	0.29	0.8
Carbohydrates (% energy)															
Pre-intervention	49.4	7.2	48.3	6.8	50.1	7.5	50	7.0	49.6	6.7	50.3	7.2	0.75	0.86	0.9
Post-intervention	50.0	7.7	49.4	7.2	50.4	8.1	49.2[4]	7.0	48.9	6.5	49.4	7.3	0.39	0.65	0.4
Fat (% energy)															
Pre-intervention	34.4	5.8	33.8	5.5	34.8	6.0	34.4	5.5	34.2	5.4	34.4	5.5	0.66	0.44	0.9
Post-intervention	34.1	5.6	33.3	5.8	34.7	5.4	35.2	5.7	35.2	5.7	35.2	5.8	0.07	0.13	0.3
SFA (% energy)															
Pre-intervention	11.9	3.2	11.8	3.1	12.0	3.3	11.7	3.0	11.7	3.3	11.6	2.8	0.4	0.76	0.4
Post-intervention	11.7	3.0	11.4	3.2	11.9	2.9	12.1[5]	2.9	11.9[6]	2.7	12.2[3]	3.1	0.3	0.48	0.5
MUFA (% energy)															
Pre-intervention	11.3	2.4	11	1.9	11.5	2.7	11.3	2.3	11.2	2.2	11.4	2.3	0.66	0.48	0.9

	Control						DHA Supplemented						Comparison of Randomized Groups (P)		
	All		***Male***		***Female***		***All***		***Male***		***Female***		***All***	***Male***	***Female***
n Pre-intervention	161		64		97		162		55		107				
n Post-intervention	137		56		81		136		50		86				
Post-intervention	11.3	2.2	10.7	2.0	11.6	2.3	11.9	2.4	11.8	2.2	11.9	2.6	0.03	0.03	0.3
***n-3* PUFA (% energy)**															
Pre-intervention	7.3	2.0	7.3	1.9	7.3	2.1	7.3	1.8	7.4	1.7	7.2	1.8	0.53	0.9	0.3
Post-intervention	0.17	0.08	7.0	1.8	7.2	2.0	7.4	1.8	7.7[5]	1.9	7.3	1.7	0.74	0.23	0.9
ALA (% energy)															
Pre-intervention	1.6	0.1	0.16	0.1	0.16	0.1	0.16	0.75	0.17	0.08	0.16	0.07	0.16	0.56	0.2
Post-intervention	0.17	0.08	0.16	0.06	0.17	0.09	0.09	0.07	0.17	0.09	0.16	0.08	0.74	0.48	0.8
DHA (% energy)															
Pre-intervention	0.08	0.08	0.07	0.08	0.08	0.1	0.09	0.07	0.07	0.07	0.08	0.08	0.74	0.9	0.6
Post-intervention	0.09[3]	0.08	0.07	0.08	0.09[7]	0.1	0.09	0.07	0.08[8]	0.07	0.09[8]	0.09	0.3	0.9	0.6
EPA (% energy)															
Pre-intervention	0.1	0.07	0.1	0.07	0.1	0.08	0.1	0.07	0.1	0.07	0.01	0.06	0.57	0.69	0.4
Post-intervention	0.1	0.08	0.08	0.07	0.1	0.09	0.11	0.09	0.12	0.11	0.1	0.07	0.3	0.08	0.8
EPA+DHA (% energy)															
Pre-intervention	0.18	0.16	0.17	0.14	0.2	0.17	0.18	0.13	0.17	0.12	0.19	0.14	0.57	0.87	0.4
Post-intervention	0.17	0.16	0.14	0.1	0.2	0.18	0.2	0.16	0.21	0.19	0.19	0.14	0.53	0.06	0.8

[1]Randomized groups were compared using student's independent samples t-test, changes from baseline were compared using student's paired samples *t*-test; [2]Values are means, SD; [3]*P* = 0.02, [4]*P* = 0.04, [5]0.009, [6]0.01, [7]0.006, [8]<0.001; All data were natural log transformed before analyses

7.4 Discussion

A high dietary intake of *n-3* LC-PUFA is thought to reduce the risk of CVD, but evidence to support its advantages in healthy populations is scarce.[328] In the present study, we investigated the effect of DHA supplementation on endothelial function and CVD risk factors and found no evidence to support a beneficial effect on these outcomes. On the contrary, DHA supplementation was found to have a small but significant negative effect on endothelial function in men. However, beneficial effects on blood pressure in men and triglyceride concentration in both sexes were shown. These data suggest that a high DHA intake does not directly impact on endothelial function and that benefits for the primary prevention of CVD operate via improvements for other CVD risk factors. A growing body of evidence supports our findings.[327, 328, 330, 331, 617-620]

Improved endothelial function is one proposed mechanism for the protective effect of *n-3* LC-PUFAs on the development of atherosclerosis.[180, 235, 243, 256, 353, 430, 434, 571-575, 578] However, most previous RCTs have been conducted in patients at high CVD risk and relatively few studies have investigated this in healthy individuals. Khan, 2003, reported that supplementation of 5g/d of tuna oil for 8 months was shown to improve endothelial function in the peripheral arteries of healthy volunteers.[243] Similarly, supplementation with 1g/d fish oil for 14 days improved both endothelial dependent and endothelial-independent vasodilatation of the brachial artery in healthy adults.[256] The present study did not support these findings. However, previous studies were small,[256, 621]did not have a parallel, RCT design,[243] investigated endothelial function in resistance vessels, which may correlate poorly with FMD of conduit vessels such as the brachial artery,[573] or were conducted in older adults likely to already have clinical signs of endothelial dysfunction.[578] However, our findings *are* consistent with a recent RCT in middle-aged adults at moderately increased CVD risk. Sanders (2011) found no effect of *n-3* LC-PUFA supplementation (up to 1.8g/d) on FMD.[253] Similarly, the MESA study, a cross-sectional study of more than 3000 adults aged 45-84 years, found higher consumption of non-fried fish was associated with a 1 SD lower brachial artery diameter in men and 0.27% smaller FMD in women.[270] Collectively, therefore, together with these previous

reports, our data support the hypothesis that, while *n-3* LC-PUFA supplementation may improve endothelial function in individuals with clinical CVD disease[574] or risk factors such as dyslipidaemia and type-2 diabetes (McVeigh, 1993)[575] there is little evidence to suggest a beneficial effect on endothelial function in healthy adults.[571]

The detrimental effect of DHA supplementation on FMD in men was an unexpected finding and requires further investigation. One possible explanation is that *n-3* LC-PUFA supplementation has been shown to increase markers of endothelial activation. For example, soluble adhesion molecule concentration increased in younger compared to older men.[234, 247] The association between fish intake and vascular function has been shown to vary by gender previously.[270] Furthermore, women metabolise *n-3* LC-PUFA differently to men. Compared with men, women have greater tissue DHA content,[622] and a higher capacity to metabolise α-linolenic acid to DHA.[623]

As in previous reports, we found no effect of DHA supplementation on vascular measures such as carotid IMT[569] and arterial stiffness.[253] There was, however, a small benefit of DHA supplementation on mean arterial blood pressure in men but not women. Although the reasons for the sex difference are uncertain, our data are consistent with earlier studies and meta-analyses,[386] which showed that low to moderate doses of DHA can lower blood pressure in healthy, normotensive individuals without changes in endothelial function or arterial stiffness.[386]

DHA supplementation reduced triglyceride concentration as reported in previous studies.[299, 569, 577] Importantly, this effect was seen in a healthy, young population with triglyceride levels in the normal range. The size of the effect (27% lower TG concentration) was similar to data from systematic reviews in older adults [569, 577] that investigated higher *n-3* LC-PUFA doses (approximately 2.7g/day).[569] The present study found similar benefits but at lower levels of supplementation that provided *n-3* LC-PUFA within a feasible dietary range.

A dose-response association between red cell *n-3* DHA and triglyceride concentration was found and a greater benefit of DHA supplementation was seen in individuals with higher pre-supplementation triglyceride levels.[299, 569, 577] The mechanisms for this effect are unknown, but lower VLDL concentration with DHA supplementation in the present study was consistent with the hypothesis that *n-3* LC-PUFAs decrease the hepatic production of triglyceride rich particles.[569]

Chapter 8

Relationships of Dietary Patterns with Vascular Structure and Function and Classical CVD Risk Factors

They claim red meat is bad for you. But I never saw a sick-looking tiger.

~Chi Chi Rodriguez

8.1 Introduction

Substantial evidence supports the hypothesis that dietary factors have protective effects on the development of CVD (**Chapter 1, section 1.6**). Endothelial dysfunction is a major contributor to atherosclerosis and an accepted independent predictor of CV events.[36, 38] Dietary factors may modulate EF either by decreasing inflammation and thereby endothelial activation or by improving endothelium-dependent FMD in healthy subjects (**Chapter 2, Section 2.1)**. Specific foods and nutrients that may improve FMD include fish and *n-3* fatty acids and a protective role of certain dietary styles has also been reported.[92, 181]

Diet also has a direct impact on classical CVD risk factors including, blood pressure,[260] dyslipidaemia[624] and insulin resistance.[79] Furthermore, classical CVD risk factors have been shown to directly impact EF.[625] The present study aims to describe dietary patterns and to investigate associations of dietary patterns and their components with vascular structure and function and CVD risk factors in a group of healthy young adults.

8.1.1 Subject Characteristics

Three hundred and twenty three participants in the *n-3*-HYP study, a RCT of DHA supplementation in healthy young adults, completed the EPIC FFQ at baseline. Principal component analysis was performed as described in **Chapter 6**, **Section 6.5.9** and three predominant dietary patterns were identified: health conscious (healthy), meat based (meaty) or high in discretionary foods (snack). The healthy pattern was characterized by high

loadings for fruit, vegetables, fish, low-fat dairy products, wholegrain foods, nuts and vegetarian foods. The meaty pattern scored high on meat and meat products, high fat dairy foods and refined carbohydrates **(Box 8-1)**. Convenient fish products, red meats, combination dishes, confectionary and savoury snacks scored highest in the snack pattern.

Box 8-1 Food Groups Characterising Dietary Patterns

Pattern 1 – "Healthy"

Green, leafy vegetables, yellow vegetables, cruciferous vegetables, alium vegetables, other vegetables; fresh fruits, citrus fruits, other fruits; oily fish, vegetarian foods, cereals and wholegrain products.

Pattern 2 – "Meaty"

Red meats, processed meats, organ meats, poultry, high fat dairy products, refined grain products, combination meals, savoury pies, tomatoes, high fat dressings and sauces, and saturated fats.

Pattern 3 – "Snack"

Convenient fish products, combination dishes, salty snacks, confectionary, biscuits and cakes, dairy desserts, refined grain products, potato products, high energy drinks, tea, and coffee.

A one-way analysis of variance (ANOVA) was carried out to explore the relationship of dietary patterns on study outcomes. Subjects were divided into 5 groups according to dietary pattern scores for further analysis. Interactions between dietary patterns and gender were examined using general linear models (GLM) with dietary pattern quintile x gender introduced as an interaction term. Where interactions between gender and dietary patterns on outcomes were identified separate analyses were conducted to investigate differing relationships of diet with outcomes in men and women.

Trends in relationships between dietary pattern scores and outcomes were analysed in GLM with dietary pattern score entered as a continuous variable. Multiple linear regression analysis was used to adjust differences in outcomes between dietary pattern score quintiles for potential confounding factors known to be associated with outcomes under investigation. Subject characteristics

according to dietary pattern quintiles are presented in **Tables 8-1 to 8-6**. Regression models are presented in **Tables 8-7 to 8-11.**

8.1.2 Sociodemographic and anthropometric characteristics

People with high scores for the healthy pattern were more likely to be female (47/64, 73% female compared with 17/64, 27% male, *P* for trend <0.001), more likely to have a degree (*P* for trend = 0.01) and less likely to be centrally obese as indicated by lower waist circumference (*P* for trend <0.002) **(Table 8-1).**

Conversely, there were more males in higher quintiles for the meaty dietary pattern (37/64, 58% male compared with 27/64, 42% female) **(Table 8-2)**. The snack dietary pattern was associated with a less healthy lifestyle suggested by a lower physical activity level for people in the highest compared with the lowest quintile, 3 hours per day compared with one hour per day, (*P* for trend = 0.001). There was also a trend towards higher waist circumference with increasing scores on the snack pattern (*P* for trend = 0.03) **(Table 8-3)**.

8.2 Dietary patterns and vascular structure and function

Common carotid artery intima media thickness decreased with increasing score on the healthy dietary pattern (*P* for trend = 0.04) **(Table 8-4)**. In secondary analyses this effect was confined to females (dietary pattern x gender interaction on CCA- IMT: *P* = 0.04). After adjustment for arterial diameter and room and skin temperature, mean CCA-IMT was 0.07 mm (12%) lower for females in the highest compared with the lowest quintile for this dietary pattern score (*P* = 0.002) **(Table 8-7)**. This effect was attenuated after further adjustment for confounders known to influence vascular structure and function (age, sex, skin temperature, room temperature, social class, physical activity, blood pressure, waist circumference, LDL cholesterol, triglycerides, fasting insulin concentrations and energy intake) but almost remained significant (*P* = 0.05) **(Table 8-7).**

In contrast, a trend towards increasing brachial arterial distensibility was seen in association with higher scores on the snack dietary pattern (*P* for trend = 0.05) **(Table 8-6)**. In secondary analyses this effect was confined to women (dietary pattern x gender interaction on brachial artery distensibility: *P* = 0.002). The effect size was large (difference between highest and lowest quintile 0.03mm (43%) [*P* = 0.003]) **(Table 8-7, Model 1)** and remained after adjustment for confounders (*P* = 0.02) **(Table 8-7, Model 2)**.

There was no association of dietary patterns with FMD or other measures of vascular function **(Tables 8-4 to 8-6)**.

8.3 Dietary patterns and CVD risk factors

CVD risk factors including obesity, blood pressure, serum cholesterol, triglycerides and insulin resistance were generally related to the three dietary patterns in the expected directions with less healthy dietary patterns associated with greater CVD risk **(Table 8.1-3)**. These are discussed in detail below.

8.3.1 Obesity

A significant negative association was seen for waist circumference and WHR with healthy dietary pattern score. As dietary pattern score increased waist circumference and WHR decreased (*P* for trend = 0.002 and <0.001 respectively) **(Table 8-1)**.

In secondary analyses waist circumference was significantly lower in all quintiles compared with the lowest quintile of healthy dietary pattern score (mean difference for -highest minus lowest quintile = 5.7 cm; 95% CI:-9.1 - -2.2 [*P* = 0.001]) **(Table 8-8, Model 1)**. After adjustment for potential confounders (age and sex and energy intake) the effect was attenuated and no longer significant (*P* for trend = 0.2) **(Table 8-8, Model 2)**.

In contrast, there was a tendency toward greater obesity risk in association with the meaty and snack dietary patterns. BMI increased by a mean of 1.2 points between the lowest and highest quintiles of the snack dietary pattern score (*P* = 0.03) **(Table 8-3)**. An increase in waist circumference and waist to hip ratio was also associated with increased scores for the snack pattern (*P* for trend = 0.03 and 0.02 respectively) **(Table 8-3)**. An increase in WHR was also seen in association with increased scores for the meaty pattern (*P* for trend = 0.003) **(Table 8-2)**. These effects were independent of gender (*P* for gender x dietary pattern score on BMI, waist circumference and waist hip ratio > 0.5 for all dietary patterns).

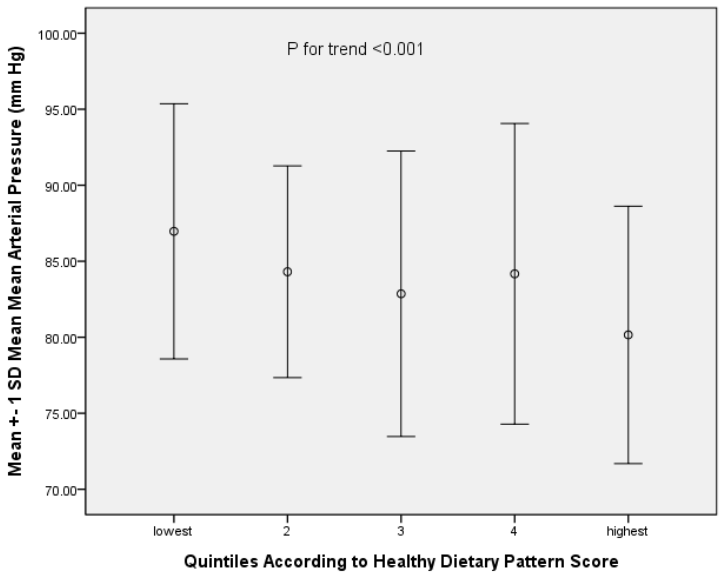
After adjustment for potential confounders (age, sex and energy intake) the effects were attenuated and no longer significant **(Table 8-8, Model 2)**.

8.3.2 Blood Pressure

Systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MAP) decreased with increasing healthy dietary pattern score (*P* for trend < 0.001 for all variables) **(Table 8-1, Figure 8.2a)**. The effect size was large (mean difference between highest and lowest quintiles of healthy dietary pattern score for MAP -6.8 mm Hg; 95% CI:-9.9 - -3.7 [*P* < 0.001]) and was consistent for

SBP, DBP and MAP **(Table 8-9, Model 1)**. There were no significant interactions of dietary patterns with gender on BP (*P* > 0.4 for all measures).

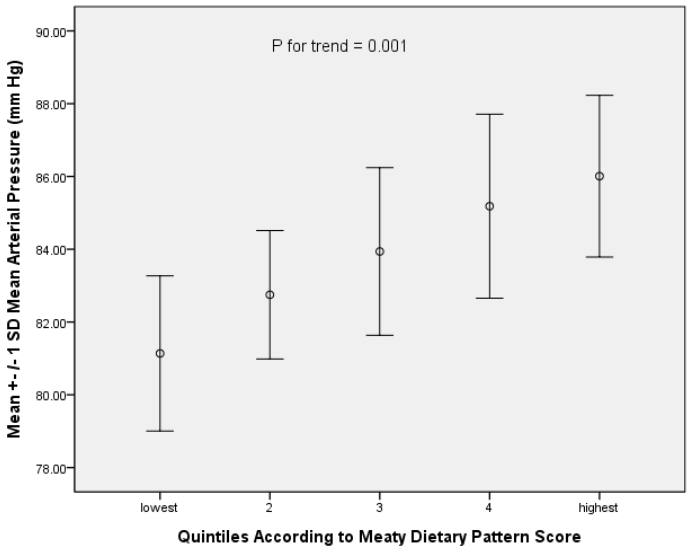
Figure 8-2a Mean (SD) arterial blood pressure according to quintiles of healthy dietary pattern score



Adjustment for potential confounding factors (age, sex, waist circumference, physical activity, social class, LDL cholesterol, triglycerides, fasting insulin and energy intake) attenuated associations of the healthy dietary pattern with BP. However, associations of MAP and DBP remained significant and a trend was seen for SBP (*P* = 0.02 , 0.002, and 0.06 respectively) **(Table 8-9, model 2)**.

The meaty dietary pattern was associated with increased BP (*P* for trend = 0.001 for all BP measures) **(Table, 8-2, Figure 8-2b)**. The mean difference for MAP between the highest and lowest quintile of meaty dietary pattern score was 5 mm Hg (95% CI: 1.8, 7.9, [*P* = 0.002]) **(Table 8-9, Model 1)**. The greatest effect was for SBP (difference for SBP between highest and lowest quintile = 7mm Hg, 95% CI: 3.2 - 10.5 [*P* < 0.001]). Adjustment for confounding factors attenuated associations for DBP and MAP slightly but all relationship between the meaty diet and BP remained significant (difference for SBP between highest and lowest quintile = 5.5mm Hg, 95% CI: 2.1 - 8.8 [*P* < 0.001]) **(Table 8-9, Model 2)**.

Figure 8-2b Mean (SD) arterial blood pressure according to quintiles of meaty dietary pattern score



8.3.3 Resting Heart Rate

Resting heart rate was lower by 6 beats per minute in the highest compared with the lowest healthy dietary pattern score quintile (95% CI:-9.3 - -2.1 [*P* = 0.002]) and higher by 5 beats per minute in the highest compared with the lowest snack dietary pattern score (95% CI: 1.5 - 8.7 [*P* = 0.006]) **(Table 8-9, Model 1)**. This effect remained after adjustment for confounding factors for both dietary patterns (*P* for trend = 0.02 and 0.04 for the healthy and snack patterns respectively) **(Table 8-9, Model 2)**.

8.3.4 Serum lipid concentrations

The healthy dietary pattern was associated with a more favourable lipid profile. Total cholesterol, LDL, VLDL and TG were lower in association with the healthy dietary pattern (*P* for trend = 0.01 for TC and LDL and *P* < 0.001 for VLDL and TG) **(Table 8-1)**. Total cholesterol was lower by 0.5mmol/L (11%) (95% CI: -0.8 - -0.2) in the highest compared with the lowest quintile (*P* = 0.002), LDL was lower by 0.4mmol/L (15%) (95% CI: -0.67 - -0.16 [*P* = 0.002]), VLDL by 0.2mmol/L (40%) (95% CI: -0.2 - -0.6 [*P* < 0.001]) and TG by 0.3mmol/L (25%) (95% CI: -0.06 - -0.1 [*P* = 0.002]) **(Table 8-10, Model 1)**. LDL cholesterol increased with higher scores on the snack dietary pattern. No significant

associations between the meaty pattern and serum lipid concentrations were found.

The lipid profile improved as healthy dietary pattern score increased. The ratio of total to HDL cholesterol decreased by 11% from 3.5 in the lowest to 3.1 in the highest healthy dietary pattern score quintile (*P* for trend = 0.003) **(Table 8-1)**. In contrast, the ratio of total to HDL cholesterol was higher for participants in the highest quintile for the snack dietary pattern (*P* for trend = 0.01) **(Table 8-3)**. The ratio of LDL (the more atherogenic lipoprotein particle) to HDL also increased as scores on the snack dietary pattern increased (*P* for trend = 0.02) **(Table 8-3)**. There were no significant interactions between dietary pattern scores and gender (dietary pattern x gender interaction: *P* > 0.1 for all variables.

Most associations of lipids with dietary patterns were attenuated and became non-significant when adjusted for potential confounding factors: age, sex, WHR, physical activity, social class, blood pressure, and energy intake. However, associations of VLDL and TG with the healthy dietary pattern still showed a trend that almost reached significance (*P* for trend = 0.06 for both variables) **(Table 8-10, Model 2).**

8.3.5 Fasting serum insulin

Serum insulin concentration was associated with the healthy and snack dietary patterns in opposite directions. Fasting insulin decreased across quintiles of healthy dietary pattern score (*P* for trend = 0.04) **(Table 8-1)** and increased with snack pattern score (*P* for trend = 0.02) **(Table 8-3)**. A similar pattern was seen for Insulin resistance (IR), as measured by HOMA. HOMA-IR score was 0.2 units (22%) greater in the highest compared with the lowest snack dietary pattern quintile (95% CI: 0.06 - 0.5 [*P* = 0.01]) **(Table 8-11, Model 1)**. Effects were attenuated and no longer significant after adjustment for potential confounding factors: age, sex, WHR, physical activity, social class, blood pressure, serum lipids, and energy intake **(Table 8-11, Model 2)**

8.3.6 C-reactive protein

Serum CRP concentration, a maker of inflammation, was associated with the snack dietary pattern (*P* for trend = 0.05) **(Table 8-3)**. CRP concentration increased with high scores on this pattern. A significant interaction between this dietary pattern with gender on CRP was found (*P* for interaction 0.04) and the effect was confined to females (*P* = 0.01). The effect size was considerable (difference between highest and lowest quintile = 2.0mg/L [*P* = 0.01]) **(Table 8-11, Model 1)**. However, the effect was attenuated and the trend was no longer significant after adjustment for potential confounding factors **(Table 8-11, Model 2).**

8.4 CVD risk factors and vascular health

This study found that dietary patterns were directly related to arterial distensibility – a measure of arterial stiffness. However, no direct relationship of diet with vascular function was apparent. Direct associations of dietary patterns and CVD risk factors were found. Therefore, correlation analyses were carried out to investigate associations of CVD risk factors with vascular structure and function.

8.4.1 Obesity and vascular health

This study found direct associations of obesity with measures of vascular structure. Waist circumference and body mass index were positively associated with CCA-IMT and negatively with right brachial and common carotid artery distensibility. Effect sizes were moderate to high ranging from *r* = 0.23 for the association of waist circumference with CCA-IMT to *r* = 0.29 for waist circumference and RCCA distensibility **(Table 8-12).**

8.4.2 Blood Pressure, heart rate and vascular health

Moderate correlations were found between blood pressure and vascular health. Systolic, diastolic and MAP were related to vascular structure. Effect sizes ranged from *r* = -0.39 for MAP with RCCA distensibility to *r* = -0.31 for MAP and

CCA-IMT. Heart rate was negatively associated with RCCA distension **(Table 8-12)**.

8.4.3 Serum lipids and vascular health

Total, LDL and VLDL cholesterol were negatively associated with RCCA distensibility (*r* = -0.17, -0.21 and -0.16 respectively). LDL cholesterol was positively associated with CCA-IMT (*r* = 0.12). TG was negatively associated with RCCA distensibility (*r* = -0.16) **(Table 8-13).**

8.4.4 Strength of associations of risk factors with vascular health

The value of CVD risk factors in prediction of coronary events is an important consideration. Therefore, regression analyses were carried out to identify the proportion of variance in vascular measures attributable to select CVD risk factors (Framingham factors). As diet is the major focus of this thesis, this analysis included relationships between dietary patterns and vascular outcomes. To allow comparisons with previous studies, the contribution of risk factors that make up the Framingham risk score was also considered.

8.4.4.1 Variance due to dietary patterns

The proportion of the variance in vascular outcomes explained by dietary patterns was quite small but nevertheless significant **(Table 8-14)**. CCA-IMT decreased by about 0.01 mm for each increase in dietary pattern score quintile. This represents a 2% decrease from the study population mean CCA-IMT **(Chapter 7, Table 7-3)**. The proportion of variance explained by this dietary pattern was 1%. Similarly, brachial artery distensibility decreased by 0.01mm for each increase in snack dietary pattern score quintile which also accounted for 1% of the variance in this outcome.

8.4.4.2 Variance due to Framingham risk factors

In multivariate analyses the total variance in vascular outcomes accounted for by Framingham factors was 20, 9 and 6% for CCA-IMT, FMD and brachial artery distensibility respectively. Two Framingham factors were excluded from this analysis, presence of diabetes and use of hypertension medication, as

these were exclusion criteria for the RCT. Univariate analyses found that age and SBP were the risk factors that accounted for most of the variance in CCA-IMT (10 and 9% respectively). Gender accounted for almost all the variation in FMD attributable to Framingham factors. FMD was 2.3% greater in women compared with men and this accounted for 9% of variance according to risk factors. Gender and age were the main factors accounting for variance in brachial artery distension. However, the snack dietary pattern contributed equally with age to the total variance (**Table 8-14**).

8.5 Summary of Results

This study found direct associations of dietary patterns with measures of vascular structure. High scores on a healthy dietary pattern were associated with lower CCA-IMT, an accepted marker of sub-clinical atherosclerosis, in women.[626] This supports findings from previous studies that have identified relationships between dietary patterns and CCA-IMT.[281, 287] These are discussed in **Chapter 2, Section 2.5.2**.

A higher score on the snack dietary pattern was associated with greater brachial artery distension in women. This finding was unexpected as the foods which characterise the snack pattern would largely be accepted as unhealthy food choices. This is discussed in **Chapter 11, Section 11.2.2**. There were no associations between dietary patterns and FMD in the present study.

Increased concentration of CRP, an inflammatory marker of endothelial dysfunction, was associated with higher scores on the snack dietary pattern in women. This supports findings from other studies where healthy dietary patterns were associated with lower CRP concentration[287] and less healthy patterns with higher concentrations.[627]

Dietary patterns were associated with classical CVD disease risk factors. Benefits for obesity, blood pressure, heart rate, lipid profile (lower concentrations of VLDL and TG) and insulin resistance were suggested by reductions in these risk factors as scores on the healthy dietary pattern

increased. In contrast, CVD risk factors increased with scores on the meat and snack dietary patterns. CVD risk factors that were related to dietary patterns in this present study were moderately correlated with measures of vascular structure. Therefore, this study suggests that diet has both direct and indirect effects on the early development of atherosclerosis.

Data from this observational study provide evidence for an association of dietary patterns in adulthood and primary development of CVD. These findings are discussed fully in **Chapter 11** where Implications for clinical practice and future research are considered.

Table 8-1: Subject Characteristics at Baseline According to Quintiles of Health Conscious Dietary Pattern Score

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Dietary Pattern Score	**64**	-1.2 (0.3)	**65**	-0.6 (0.1)	**65**	-0.1 (0.1)	**65**	0.4 (0.1)	**64**	1.5 (0.8)	
Males	**39**	-1.2 (0.3)	**27**	-0.6 (0.2)	**19**	-0.1(0.1)	**18**	-0.4 (0.2)	**17**	1.6 (1.1)	
Females	**25**	-1.1 (0.2)	**38**	-0.6 (0.1)	**46**	-0.1 (0.1)	**47**	-0.4 (0.1)	**47**	1.5 (0.7)	
Gender (% male)		61		42		29		28		27	***<0.001***
Age, y		28.7 (4.8)		27.8 (5.1)		27.9 (4.9)		27.3 (4.7)		27.8 (4.2)	0.2
Males		29.1 (4.8)		28.9 (4.9)		28.9 (4.2)		28.2 (4.2)		29.6 (4.3)	0.9
Females		28.1 (4.7)		27 (5.1)		28 (4.7)		26.9 (4.9)		(27.1) (3.9)	0.5
[1]**Education:**											
with degree, %,(n)		60 (38)		59 (38)		74 (48)		68 (44)		71 (45)	***0.01***
Males		61 (23)		59 (16)		68 (13)		61 (11)		71 (12)	***0.01***
Females		72 (15)		70 (22)		76 (35)		70 (33)		72 (33)	***0.02***
[1]**Social class:**											
Non-manual, %, (n)		55 (36)		55 (36)		57 (37)		57 (37)		59 (38)	0.7
Males		49 (19)		67 (18)		63 (12)		50 (9)		53 (9)	0.9
Females		68 (17)		34 (18)		54 (25)		60 (28)		62 (29)	0.7
[1]**Current smoker,%, n**		13 (8)		12 (8)		20 (13)		9 (6)		13 (8)	0.5
Males		46 (18)		15 (4)		32 (6)		28 (5)		47 (8)	0.7
Females		48 (12)		57 (21)		41 (19)		40 (19)		32 (15)	0.7
[2]**Cotinine, ng/ml**		2.6 (284)		0.8 (219)		3 (279)		1.4 (243)		1.4 (237)	0.2
Males		0.5 (275)		0.7 (248)		4.4 (311)		1.3 (246)		2.8 (2.0)	0.8
Females		4.8 (302)		0.9 (201)		2.6 (269)		1.4 (243)		1.2 (218)	0.2
Physical activity level, hours/day		1.08 (1.6)		1.54 (1.9)		1.63 (1.8)		1.58 (2.1)		2.44 (2.1)	0.1

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Males		1.3 (1.6)		1.7 (1.9)		2.6 (2)		3.3 (2.6)		2.8 (2)	0.2
Females		0.7 (1.7)		1.4 (1.9)		1.24 (1.6)		0.9 (1.5)		2.34 (2.2)	***0.003***
Anthropometry and body composition											
Body Mass Index, kg/m^2		24.6 (3.7)		23 (22.8)		23.2 (4.1)		23.5 (3.7)		23.8 (4.3)	0.5
Males		24.9 (3)		23.4 (3)		23.4 (2.9)		24 (3.1)		24.6 (2.7)	0.5
Females		24.1 (4.7)		22.7 (4.3)		23.1 (4.5)		23.3 (4)		23.5 (4.7)	1.0
Waist (cm)		81.9 (10.6)		77.6 (12.4)		75.4 (8.6)		75.8 (8.8)		76.2 (9)	***0.002***
Males		85.3 (9)		83.1 (8.9)		82.5 (7.9)		80.1 (7.4)		84.4 (7.4)	0.3
Females		76.6 (10.9)		73.5 (13.1)		72.4 (7.1)		74.2 (9)		73.2 (7.6)	0.4
Waist:Hip ratio		0.82 (0.07)		0.78 (0.07)		0.76 (0.06)		0.77 (0.07)		0.77 (0.06)	***<0.001***
Males		0.85 (0.07)		0.83 (0.05)		0.83 (0.05)		0.83 (0.05)		0.83 (0.05)	0.3
Females		0.78 (0.05)		0.75(0.07)		0.73 (0.04)		0.74 (0.05)		0.74 (0.04)	0.09
[2]Sum skinfold, mm		53.3 (22.4)		46.9 (17.7)		51.0 (18.8)		53.1 (22.1)		52.8 (20.1)	0.5
Males		45.1 (41.3)		40 (39.4)		39 (41.5)		35.2 (39.2)		39.7 (41.9)	0.08
Females		54.7 (47.5)		46.4 (37.1)		51.5 (36.8)		54.9 (39.4)		52.9 (36.2)	0.6
% Fat (skinfolds)		23.9 (4.9)		25.2 (6.3		24.4 (5.2)		23.4 (6.1)		23 (5.2)	0.1
Males		23.1 (5)		22.7 (6.8)		23.1 (4.9)		24.4 (5.0))		23.5 (6)	0.6
Females		25 (4.4)		26.4 (6.4)		24.9 (5.3)		23 (6.5)		22.8 (4.9)	***0.004***
Blood pressure, mm Hg											
Systolic	**63**	115 (10)	**65**	113 (9)	**65**	111 (11)	**65**	112 (12)	**63**	108 (11)	***<0.001***
Males	**38**	118 (10)	**27**	117 (9)	**19**	117 (10)	**18**	116 (7)	**17**	111 (12)	***0.05***
Females	**25**	110 (9)	**38**	110 (7.9)	**46**	109 (10)	**47**	111 (13)	**46**	106 (10)	0.1
Diastolic		69 (7)		68 (6)		66 (8)		68 (9)		63 (7)	***<0.001***
Males		70 (8)		70 (7)		69 (9)		68 (7)		64 (6)	***0.02***
Females		68 (7)		67 (6)		64 (8)		67 (9)		63 (7)	***0.008***

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Mean arterial pressure		87 (8)		85 (6)		83 (9)		84 (10)		80 (9)	***<0.001***
Males		89 (9)		88 (5)		87 (11)		87 (7)		84 (8)	***0.05***
Females		84 (8)		83 (6)		81 (8)		83 (11)		79 (9)	***0.04***
Resting heart rate, beats/min		68 (10)		68 (13)		66 (9)		98 (11)		62 (10)	***0.02***
Males		66 (11)		67 (16)		62 (9)		69 (12)		59 (8)	0.3
Females		72 (7)		68 (9)		67 (9)		68 (11)		63 (11)	***0.001***
†**Total cholesterol, mmol/L**	**61**	4.5 (0.9)	**64**	4.1 (0.9)	**65**	4.2 (0.7)	**64**	4.3 (0.9)	**63**	4.1 (0.7)	***0.01***
Males	37	4.5 (0.9)	26	4.3 (0.8)	19	4.2 (0.6)	18	4.4 (1)	17	4.0 (0.6)	***0.02***
Females	24	4.5 (0.8)	38	4 (0.9)	45	4.2 (0.9)	46	4.2 (0.8)	47	4.1 (0.7)	0.3
†LDL cholesterol, mmol/L		2.6 (0.8)		2.3 (0.7)		2.3 (0.7)		2.4 (0.8)		2.2 (0.6)	***0.01***
Males		2.7 (0.8)		2.6 (0.9)		2.4 (0.7)		2.7 (0.8)		2.3 (0.5)	0.2
Females		2.5 (0.9)		2.1 (0.6)		2.2 (0.8)		2.3 (0.8)		2.2 (0.7)	0.3
†**HDL cholesterol, mmol/L**		1.3 (0.3)		1.5 (0.4)		1.5 (0.4)		1.5 (0.3)		1.5 (0.4)	0.08
Males		1.2 (0.2)		1.4 (0.3)		1.4 (0.3)		1.3 (0.3)		1.3 (0.3)	0.5
Females		1.5 (0.4)		1.5 (0.4)		1.5 (0.4)		1.6 (0.3)		1.5 (0.3)	1.0
†[2]**VLDL cholesterol**		0.5 (0.3)		0.5 (0.3)		0.4 (0.3)		0.4 (0.2)		0.4 (0.2)	***<0.001***
Males		0.5 (47)		0.4 (37)		0.4 (66)		0.4 (50)		0.3 (59)	***0.01***
Females		0.4 (40)		0.4 (49)		0.4 (44)		0.4 (45)		0.3 (50)	0.2
Total:HDL cholesterol ratio		3.5 (1.1)		3.0 (1.0)		3.1 (1.1)		2.9 (0.8)		3.1 (1.0)	***0.003***
Males		3.8 (1)		3.4 (1.1)		3.5 (1.1)		3.4 (0.6)		3.3 (1.0)	0.07
Females		3.1 (1.1)		2.7 (0.8)		2.9 (1)		2.8 (0.9)		2.8 (0.7)	0.5
LDL:HDL cholesterol ratio		1.7 (0.7)		1.7 (0.8)		1.7 (0.9)		1.7 (0.8)		2.1 (0.9)	***0.008***
Males		2.3 (0.8)		2 (1)		2 (0.9)		2.1 (0.6)		2 (0.8)	0.2
Females		1.8 (1)		1.5 (0.6)		1.6 (0.8)		1.6 (0.8)		1.5 (0.6)	0.4
†[2]**Trigycerides, mmol/L**		1.2 (0.7)		1.0 (0.6)		1.0 (0.7)		0.0 (0.4)		0.9 (0.5)	***<0.001***

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Males		0.9 (59)		0.8 (50)		0.9 (66)		0.8 (37)		0.7 (47)	***0.01***
Females		0.9 (40)		0.9 (44)		0.8 (49)		0.8 (45)		0.8 (50)	0.2
[†2]Glucose, mmol/L		4.9 (0.5)		4.9 (0.4)		4.8 (0.5)		4.8 (0.5)		4.7 (0.4)	***0.006***
Males		5.0 (0.5)		5.0 (0.5)		4.8 (0.4)		4.9 (0.4)		5.0 (0.3)	0.4
Females		4.7 (0.4)		4.8 (0.4)		4.7 (0.6)		4.7 (0.5)		4.6 (0.4)	0.2
[2†]Insulin, pmol/L		28.5 (62)		28.5 (58)		29.9 (62)		28.5 (53)		27.8 (63)	***0.04***
Males		34.8 (63)		26.4 (59)		28.5 (47)		23 (48)		26.4 (57)	***0.03***
Females		28.5 (61)		38.9 (57)		30.6 (67)		31.3 952)		28.5 (63)	0.1
[2]Insulin resistance, (HOMA)		0.9 (69)		0.9 (60)		0.9 (67)		0.8 (59)		0.8 (61)	***0.03***
Males		0.9 (72)		0.8 (64)		0.8 (52)		0.7 (52)		0.8 (56)	***0.05***
Females		0.8 (66)		0.9 (57)		0.9 (73)		0.9 (59)		0.8 (63)	0.2
[2]CRP, mg/L		1.0 (127)		1.0 (105)		1.1 (135)		1.1 (120)		0.9 (115)	0.8
Males		0.7 (114)		0.7 (96)		0.9 (136)		0.5 (118)		1 (120)	0.2
Females		0.9 (133)		1.2 (109)		1.2 (135)		1.2 (122)		0.9 (116)	0.3

* Quintile 1 is the lowest quintile for dietary pattern score. ‡ *P* for trend across quintiles calculated with the dietary pattern score quintile modelled on a continuous scale in general linear models. Data are mean, SD, except: [1] % (n) and [2] geometric mean (coefficient of variation). [†] Measurements obtained after 12 hours fasting.

Table 8-2: Subject Characteristics at Baseline According to Quintiles of Meat Based Dietary Pattern Score

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Dietary Pattern Score	**64**	-1 (0.3)	**65**	-0.5 (0.1)	**65**	-0.2 (0.1)	**65**	0.2 (0.2)	**64**	1.4 (1.3)	
Males	**14**	-0.9 (0.2)	**18**	-0.5 (0.1)	**25**	-0.2 (0.09)	**26**	0.2 (0.09)	**37**	1.3 (0.6)	
Females	**50**	-1 (0.3)	**47**	-0.5 (0.1)	**40**	-0.2 (0.09)	**39**	0.2 (0.2)	**27**	1.5 (1.9)	
Gender (% male)		22		28		38		40		58	***<0.001***
Age, y		27.7 (4.9)		28.3 (4.5)		27.8 (5)		28.2 (4.4)		27.4 (4.8)	0.6
Males		30.5 (4.9)		30 (5.1)		28.2 (4.7)		29.2 (3.7)		28.2 (5.1)	0.1
Females		27 (4.6)		27.6 (4.2)		27.6 (5.2)		27.5 (4.8)		26.3 (4.3)	0.7
[1]Education:											
with degree, %, n		66 (42)		81 (53)		59 (37)		62 (40)		65 (41)	0.2
Males		64 (9)		78 (14)		56 (14)		69 (18)		56 (20)	0.3
Females		66 (33)		83 (39)		61 (23)		56 (22)		78 (21)	0.7
[1]Social class:											
Non-manual, %, n		55 (35)		56 (36)		58 (38)		52 (34)		58 (38)	0.8
Males		50 (7)		50 (9)		52 (13)		54 (14)		65 (24)	0.3
Females		58 (29)		62 (29)		52 (13)		54 (14)		65 (24)	0.7
[1]Current Smoker, %, n		13 (8)		9 (6)		13 (8)		14 (9)		19 (12)	0.4
Males		10 (6)		12 (3)		16 (4)		0.05 (1)		0	0.9
Females		26 (7)		18 (7)		13 (5)		11 (5)		16 (8)	0.09
[2]Cotinine, ng/ml	38	26.1 (97.3)	42	22.2 (80.2)	39	16.0 (48.6)	39	22.5 (57.5)	39	37 (95.4)	0.5
Males		0.9 (1.5)		1.7 (3)		1.1 (3)		2.2 (3.2)		2.5 (2.9)	0.3
Females		1.9 (253)		1 (213)		1.2 (247)		2.1 (244)		4.4 (278)	0.7
Physical activity level, hours/day	63	1.6 (2.1)	65	1.3 (1.6)	65	2.1 (2.2)	65	1.8 (1.9)	63	1.5 (2.0)	0.6

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Males		1.9 (1.8)		2 (1.8)		2.6 (2.3)		2.3 (1.9)		1.8 (2.2)	0.3
Females		1.5 (2.1)		1.1 (1.5)		1.8 (2.1)		1.4 (1.9)		1.1 (1.6)	0.8
Anthropometry and body composition											
Body Mass Index, kg/m^2	**64**	23.3 (3.7)	**65**	23.8 (3.3)	**65**	24.2 (4.7)	**65**	23.9 (4.5)	**64**	22.8 (3.2)	0.6
Males	**14**	24 (3.6)	**18**	24.1 (2.1)	**25**	25 (2.7)	**26**	24.8 (3.2)	**37**	223.2 (3.1)	0.3
Females	**50**	23.1 (3.7)	**47**	23.7 (3.7)	**40**	23.8 (5.6)	**39**	23.3 (5.1)	**27**	22.1 (3.4)	0.5
Waist (cm)		75.5 (8.6)		76.8 (8.9)		79.3 (12.2)		77.8 (11.3)		77.3 (9)	0.2
Males		83.4 (10.3)		83.4 (7.2)		84.8 (6.7)		85 (8.4)		81.2 (8.3)	0.3
Females		73.2 (6.5)		74.2 (8.2)		75.8 (13.6)		73 (10.5)		72.1 (7)	0.5
Waist:Hip ratio		0.76 (0.07)		0.77 (0.6)		0.78 (0.07)		0.78 (0.07)		0.79 (0.07)	***0.003***
Males		0.84 (0.07)		0.82 (0.05)		0.84 (0.05)		0.85 (0.06)		0.83 (0.05)	0.9
Females		0.71 (0.05)		0.74 (0.04)		0.77 (0.07)		0.74 (0.04)		0.74 (0.05)	0.9
[2]Sum skinfold, mm		52.1 (20.8)		54.3 (23.2)		50.0 (18.2)		54.2 (20.1)		46.3 (19.0)	0.1
Males		36.5 (36)		40.4 (37)		44.8 (38)		44 (38)		37.5 (36)	0.8
Females		51.4 (39)		53 (45)		48 (36)		55.1 (35)		51 (35)	0.9
% Fat (skinfolds)		24.1 (5.9)		24.4 (5.8)		24.8 (4.9)		23.5 (5.4)		23.1 (5.8)	0.2
Males		23.3 (5.7)		24 (5.8)		24.1 (5.2)		23 (4.5)		23 (5.5)	0.6
Females		24.3 (6)		24.3 (5.8)		25.3 (4.8)		23.8 (6)		23 (6.4)	0.4
Blood pressure, mm Hg											
Systolic	**64**	108 (10.6)	**65**	111 (9)	**65**	112 (10)	**64**	114 (12)	**63**	115 (10)	***0.001***
Males	**14**	114 (14)	**18**	114 (8)	**25**	114 (6)	**26**	120 (9)	**36**	119 (10)	***0.01***
Females	**50**	106 (9)	**47**	109 (9)	**40**	110 (12)	**38**	111 (12)	**27**	110 (11)	0.06
Diastolic		81 (8)		83 (7)		84 (9)		85 (10)		86 (9)	***0.001***
Males		67 (10)		66 (7)		68 (6)		70 (8)		70 (8)	0.06

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Females		64 (7)		65 (7)		67 (9)		65 (9)		66 (8)	0.2
Mean arterial pressure		81 (8)		83 (7)		84 (9)		85 (10)		86 (9)	***0.001***
Males		86 (10)		85 (7)		85 (6)		89 (9)		89 (8)	0.06
Females		80 (7)		82 (7)		83 (11)		83 (10)		82 (9)	0.1
Resting heart rate, beats/min)		64 (10)		67 (10)		67 (11)		68 (10)		66 (10)	0.1
Males		60 (6)		63 (11)		65 (9)		64 (9)		65 (10)	0.1
Females		65 (9)		67 (9)		68 (9)		69 (10)		65 (7)	0.09
†**Total cholesterol, mmol/L**	**64**	4.3 (1.0)	**62**	4.1 (1.0)	**64**	4.3 (0.7)	**64**	4.2 (0.8)	**63**	4.2 (0.8)	0.7
Males	**14**	4.5 (0.8)	**16**	4.2 (0.9)	**25**	4.2 (0.7)	**26**	4.5 (1)	**36**	4.3 (0.9)	0.9
Females	**50**	4.3 (0.9)	**46**	4.1 (1)	**39**	4.3 (0.7)	**38**	4.1 (0.7)	**27**	4.1 (0.7)	0.4
†**LDL cholesterol, mmol/L**		2.4 (0.9)		2.3 (0.7)		2.4 (0.7)		2.4 (0.8)		2.3 (0.7)	0.8
Males		2.7 (0.7)		2.5 (0.7)		2.5 (0.7)		2.7 (0.8)		2.5 (0.8)	0.6
Females		2.3 (0.9)		2.1 (0.7)		2.4 (0.7)		2.2 (0.7)		2.1 (0.7)	0.3
†**HDL cholesterol, mmol/L**		1.5 (0.4)		1.4 (0.3)		1.4 (0.3)		1.4 (0.3)		1.47 (0.3)	0.4
Males		1.3 (0.3)		1.2 (0.3)		1.2 (0.2)		1.3 (0.4)		1.4 (0.3)	0.4
Females		1.6 (0.4)		1.5 (0.3)		1.5 (0.4)		1.5 (0.3)		1.6 (0.3)	0.09
[2]**VLDL cholesterol**		0.4 (0.2)		0.5 (0.3)		0.4 (0.2)		0.5 (0.3)		0.4 (0.4)	0.7
Males		0.4 (63)		0.4 (45)		0.4 (46)		0.5 (49)		0.4 (65)	0.6
Females		0.4 (46)		0.4 (50)		0.4 (45)		0.4 (47)		0.4 (43)	0.5
Total:HDL cholesterol ratio		3.0 (1.1)		3.0 (1.0)		3.3 (1.0)		3.2 (1.1)		3.0 (0.9)	0.5
Males		3.5 (1)		3.7 (1.1)		3.5 (0.8)		3.8 (1.2)		3.3 (1)	0.5
Females		2.9 (1.1)		2.7 (0.7)		3.1 (1.1)		2.8 (0.8)		2.6 (0.6)	0.5
LDL:HDL cholesterol ratio		1.7 (0.7)		1.8 (0.9)		1.9 (0.8)		1.7 (0.7)		1.7 (0.9)	0.8
Males		2.1 (0.8)		2.2 (0.9)		2.1 (0.7)		2.3 (1)		1.9 (0.7)	0.3
Females		1.4 (0.5)		1.5 (0.7)		1.8 (0.9)		1.5 (0.5)		1.6 (0.9)	0.4

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
[†2]**Trigycerides, mmol/L**		0.9 (0.5)		1.0 (0.7)		1.0 (0.4)		1.0 (0.6)		1.0 (0.8)	0.6
Males		0.8 (55)		0.9 (63)		0.9 (46)		1.1 (0.5)		0.8 (0.7)	0.6
Females		0.8 (46)		0.9 (50)		0.8 (45)		0.8 (47)		0.8 (43)	0.6
[†2]**Glucose, mmol/L**		4.7 (0.5)		4.7 (0.5)		4.8 (0.6)		4.8 (0.5)		4.9 (0.4)	0.03
Males		4.9 (0.4)		4.9 (0.4)		5.1 (0.6)		4.9 (0.5)		5.0 (0.5)	0.6
Females		4.7 (0.5)		4.7 (0.5)		4.7 (0.4)		4.7 (0.5)		4.8 (0.3)	0.4
[†2]**Insulin, pmol/L**		28.5 (64)		28.5 (50)		29.2 (65)		31.3 (67)		28.5 (50)	0.9
Males		28.5 (63)		29.9 (40)		28.5 (63)		29.9 (65)		25 (55)	0.5
Females		27.8 (65)		28.5 (53)		33.4 (66)		34 (68)		34 (36)	0.4
[2]**Insulin resistance, (HOMA)**		0.9 (69)		0.9 (53)		1 (69)		1 (71)		0.9 (54)	0.8
Males		0.9 (64)		0.9 (42)		0.9 (71)		0.9 (73)		0.8 (61)	0.7
Females		0.8 (71)		0.8 (57)		1 (69)		01.1 (70)		1 (39)	0.5
[†2]**CRP, mg/L**		1.1 (1.7)		2.1 (4.6)		1.9 (2.7)		2.0 (4.3)		1.6 (2.8)	0.6
Males		0.9 (0.9)		0.8 (1.1)		0.8 (1.4)		0.8 (1.2)		0.8 (1.1)	0.7
Females		0.8 (114)		1.1 (14)		1.5 (109)		1.1 (128)		1.1 (120)	0.3

* Quintile 1 is the lowest quintile for dietary pattern score. ‡ *P* for trend across quintiles calculated with the dietary pattern score modeled on a continuous scale in general linear models. Data are mean, SD, except: [1]% (n) and [2]geometric mean (coefficient of variation).[†] Measurements obtained after 12 hours fasting.

Table 8-3: Subject Characteristics at Baseline According to Quintiles of Snack Dietary Pattern Score

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	*P* ‡
Dietary Pattern Score	**64**		**65**		**65**		**65**		**64**		
Males	**20**	-1 (0.2)	**20**	-0.5 (0.09)	**37**	-0.2 (0.1)	**21**	0.2 (0.1)	**22**	1.2 (0.7)	
Females	**44**	-1.05 (0.3)	**45**	-0.5 (0.1)	**28**	-0.2 (0.1)	**44**	0.2 (0.1)	**42**	1.7 (1.1)	
[1]**Male gender, %, (n)**		31 (20)		31 (20)		57 (37)		32 (21)		34 (22)	0.6
Age, y		27.4 (4.8)		27.3 (4.6)		29.2 (4.5)		27.1 (4.7		8.6 (4.8)	0.2
Males		28 (5.1)		28.2 (5.2)		29.8 (4.2)		29.5 (4.5)		28.6 (5)	0.4
Females		27.1 (4.7)		26.9 (4.3)		28.3 (4.8)		26 (4.5)		28.6 (4.7)	0.4
[1]**Education:**											
with degree, %, (n)		61 (39)		71 (45)		72 (46)		62 (40)		67 (64)	0.1
Males		60 (12)		70 (14)		64 (23)		52 (11)		68 (15)	0.5
Females		41 (27)		72 (31)		82 (23)		66 (29)		67 (28)	0.2
[1]**Social class:**											
Non-manual, %, (n)		53 (34)		52 (33)		62 (40)		52 (33)		69 (44)	0.09
Males		60 (12)		55 (11)		59 (22)		52 (11)		50 (11)	0.6
Females		50 (22)		49 (22)		54 (18)		50 (22)		79 (33)	***0.02***
[1] **Current Smoker , %, (n)**		21 (13)		13 (8)		9 (6)		14 (9)		2 (7)	***0.004***
Males		30 (6)		30 (6)		38 (14)		29 (6)		41 (9)	***0.004***
Females		50 (15)		50 (15)		57 (16)		39 (17)		29 (12)	0.2
[2]**Cotinine, ng/ml**		30 (82.5)		15.2 (74.3)		17.2 (50)		29.2 (87.2)		31.5 (88.2)	0.6
Males		3.3 (289)		1.6 (297)		1 (263)		1.2 (249)		2.2 (298)	0.4
Females		3.4 (253)		0.7 (161)		1.7 (245)		2.5 (276)		1.3 (267)	0.2
Physical activity level, hours/day		2.9 (5.8)		1.8 (1.7)		1.8 (2.1)		1.4 (1.6)		1.1 (1.7)	***0.001***
Males		2.85 (2.1)		2.4 (1.9)		2.4 (2.3)		1.7 (1.6)		1.1 (1.7)	0.008

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	*P* ‡
Females		2 (2.4)		1.5 (1.5)		1 (1.5)		1.3 (1.7)		1 (1.7)	***0.01***
Anthropometry and body composition	**64**		**65**		**65**		**65**		**64**		
Males	**20**		**20**		**37**		**21**		**22**		
Females	**44**		**45**		**28**		**44**		**42**		
Body Mass Index, kg/m^2		22.8 (2.7)		23.2 (3.0)		23.6 (3.7)		24.3 (5.2)		24 (4.4)	***0.03***
Males		23.8 (2.5)		23.7 (2.6)		23.8 (2.9)		25.5 (3.3)		24.1 (3.4)	0.3
Females		22.4 (2.7)		22.9 (3.2)		23.4 (4.6)		23.8 (5.9)		23.9 (4.9)	0.07
Waist (cm)		75.5 (8.4)		75.4 (8.4)		79.2 (9.1)		78 (11.3)		78.7 (12.4)	***0.03***
Males		83.2 (10.2)		81.5 (6)		82.7 (7.3)		86.7 (8.3		83.7 (9.9)	0.1
Females		72.6 (6.7)		72.7 (7.8)		74.3 (9)		73.5 (9.9)		76 (12.8)	0.1
Waist:Hip ratio		0.7 (0.07)		0.7 (0.07)		0.8 (0.06)		0.8 (0.08)		0.8 (0.07)	***0.02***
Males		0.8 (0.05)		0.8 (0.04)		0.8 (0.04)		0.8 (0.06)		0.8 (0.07)	***0.05***
Females		0.7 (0.05)		0.7 (0.05)		0.8 (0.05)		0.7 (0.05)		0.8 (0.05)	0.07
[2]Sum skinfold, mm		48.6 (17.6)		52 (16)		45.7 (18.3)		56.7 (23.2)		53.8 (24.5)	0.07
Males		40.4 (42)		41.7 (43)		36.6 (39)		50.4 (40)		37.7 (49)	0.06
Females		47.5 (36)		53 (32)		51 (38)		52.5 (46)		54.6 (41)	0.5
% Fat (skinfolds)		22.8 (5.4)		24.5 (5.2)		23.6 (6.1)		24.3 (5.5)		24.7 (5.6)	0.1
Males		22.2 (5.6)		22.3 (4.3)		23.9 (5.7)		24.9 (5.1)		23.6 (5.2)	0.2
Females		23.2 (5.4)		25.5 (5.2)		23.3 (6.8)		24 (5.7)		25.3 (5.8)	0.3
Blood pressure, mm Hg											
Systolic	**64**	111 (9)	**64**	109 (10)	**64**	113 (10)	**65**	113 (11)	**64**	113 (13)	0.06
Males	**20**	118 (8)	**20**	112 (11)	**36**	116 (9)	**21**	118 (7)	**22**	119 (12)	0.2
Females	**44**	108 (8)	**44**	108 (9)	**28**	109 (10)	**44**	111 (11)	**42**	110 (13)	0.2
Diastolic		66 (6)		65 (7)		68 (8)		68 (8)		68 (9)	***0.02***

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	*P* ‡
Males		68 (6)		66 (7)		68 (8)		70 (6)		70 (10)	0.1
Females		64 (6.3)		64 (8)		67 (8)		67 (8)		66 (9)	0.1
Mean arterial pressure		83 (7)		81 (8)		85 (9)		85 (8.0)		85 (11)	***0.02***
Males		88 (6)		84 (7)		87 (9)		88 (6)		90 (10)	0.1
Females		81 (6.9)		80 (8)		82 (9)		83 (9)		83 (11)	0.1
Resting heart rate, beats/min		64 (10)		65 (89)		66 (10)		67 (10)		69 (12)	***0.003***
Males		62 (7)		63 (7)		64 (9)		63 (9)		67 (12)	***0.05***
Females		64 (9)		67 (7)		66 (9)		67 (8)		69 (11)	***0.02***
†**Total cholesterol, mmol/L**	**62**	4.1 (0.8)	**64**	4.2 (0.8)	**64**	4.2 (0.7)	**65**	4.2 (0.9)	**62**	4.4 (0.9)	0.1
Males	**19**	4.1 (0.8)	**20**	4.3 (0.7)	**36**	4.3 (0.7)	**21**	4.5 (0.9)	**21**	4.4 (1.1)	0.5
Females	**43**	4.1 (0.8)	**44**	4.1 (0.8)	**28**	4.2 (0.8)	**44**	4 (0.9)	**41**	4.5 (0.8)	0.1
†**LDL cholesterol, mmol/L**		2.2 (0.7)		2.3 (0.7)		2.4 (0.7)		2.3 (0.8)		2.5 (0.8)	***0.03***
Males		2.5 (0.8)		2.5 (0.8)		2.5 (0.7)		2.7 (0.8)		2.5 (0.8)	0.6
Females		2.1 (0.7)		2.2 (0.7)		2.3 (0.8)		2.1 (0.8)		2.5 (0.7)	***0.02***
†**HDL cholesterol, mmol/L**		1.4 (0.4)		1.5 (0.4)		1.3 (0.3)		1.4 (0.3)		1.4 (0.3)	***0.05***
Males		1.2 (0.3)		1.4 (0.3)		1.3 (0.3)		1.3 (0.3)		1.2 (0.2)	1.0
Females		1.6 (0.4)		1.6 (0.4)		1.4 (0.3)		1.5 (0.3)		1.5 (0.3)	***0.02***
†[2]**VLDL cholesterol**		0.4 (0.2)		0.4 (0.3)		0.4 (0.3)		0.4 (0.2)		0.5 (0.3)	0.2
Males		0.4 (40)		0.4 (52)		0.4 (59)		0.4 (48)		0.5 (70)	0.1
Females		0.4 (45)		0.4 (49)		0.4 (40)		0.4 (46)		0.4 (50)	0.6
LDL:HDL cholesterol ratio		1.6 (0.8)		1.6 (0.7)		1.9 (0.9)		1.8 (0.9)		1.9 (0.7)	***0.02***
Males		2.2 (0.8)		1.9 (0.8)		2.1 (0.8)		2.2 (0.9)		2.1 (0.7)	0.8
Females		1.4 (0.6)		1.5 (0.7)		1.7 (1)		1.6 (0.8)		1.8 (0.7)	***0.009***
Total:HDL cholesterol ratio		3.0 (0.9)		2.8 (0.9)		3.3 (1.1)		3.1 (1.1)		3.3 (1.0)	***0.01***
Males		3.6 (0.9)		3.2 (0.9)		3.5 (1)		3.6 (1.1)		3.7 (1.1)	0.5

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	*P* ‡
Females		2.7 (0.7)		2.7 (0.8)		3 (1.2)		2.9 (1)		3.1 (0.8)	***0.009***
†[2]**Trigycerides, mmol/L**		1.0 (0.5)		1.0 (0.6)		1.0 (0.7)		1.0 (0.5)		1.1 (0.7)	0.2
Males		0.9 (40)		0.8 (52)		0.9 (59)		0.9 (48)		1.1 (70)	0.1
Females		0.9 (0.5)		0.8 (0.5)		0.8 (0.4)		0.9 (0.5)		0.9 (0.5)	0.6
†[2]**Glucose, mmol/L**		4.7 (0.4)		4.8 (0.46)		4.8 (0.48)		4.9 (0.62)		4.8 (0.43)	0.1
Males		4.9 (0.5)		4.9 (0.3)		4.9 (0.4)		5.3 (0.7)		5 (0.4)	0.06
Females		4.7 (0.4)		4.7 (0.5)		4.8 (0.6)		4.7 (0.5)		4.7 (0.4)	0.6
†[2]**Insulin, pmol/L**		25.7 (62)		27.8 (55)		25 (59)		36.8 (61)		33.4 (52)	0.02
Males		25 (44)		25 (55)		23.6 (51)		40.3 (68)		32 (57)	***0.03***
Females		25.7 (69)		29.9 (55)		28.5 (67)		36 (57)		35 (50)	0.2
[2]**Insulin resistance, (HOMA)**		0.8 (65)		0.8 (58)		0.8 (63)		1.1 (69)		1.0 (56)	***0.03***
Males		0.8 (50)		0.8 (58)		0.7 (53)		1.3 (81)		1 (59)	***0.04***
Females		0.8 (71)		0.9 (58)		0.9 (73)		1.1 (62)		1.1 (54)	0.2
†[2]**CRP, mg/L**		1.3 (2.3)		1.5 (2.8)		1.6 (2.8)		1.8 (3.0)		2.5 (5.3)	***0.05***
Males		0.9 (116)		0.8 (130)		0.7 (130)		0.9 (86)		0.8 (111)	0.5
Females		0.7 (115)		1.1 (117)		1.1 (119)		1.2 (132)		1.4 (127)	***0.01***

* Quintile 1 is the lowest quintile for dietary pattern score. ‡ *P* for trend across quintiles calculated with the dietary pattern score modeled on a continuous scale in general linear models. Data are mean, SD, except: [1]% (n) and [2]geometric mean (coefficient of variation). † Measurements obtained after 12 hours fasting.

Table 8-4 Vascular Variables at Baseline According to Quintiles of Health Conscious Dietary Pattern Score

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Right Brachial Artery											
Diameter, mm	**63**	3.4 (0.6)	**65**	3.3 (0.6)	**63**	3.1 (0.5)	**65**	3.1 (0.5)	**61**	3.1 (0.6)	***0.001***
Males	**38**	3.7 (0.4)	**27**	3.8 (0.4)	**19**	3.7 (0.4)	**18**	3.7 (0.4)	**16**	3.8 (0.6)	***0.02***
Females	**25**	2.9 (0.4)	**38**	2.9 (0.4)	**44**	2.9 (0.3)	**47**	2.9 (0.4)	**45**	2.9 (0.3)	0.07
Reactive hyperaemia, %		648 (240)		769 (257)		745 (232)		742 (248)		776 (324)	**0.03**
Males		607 (231)		656 (195)		694 (25)		618 (182)		639 (277)	0.7
Females		713 (245)		840 (268)		766 (224)		292 (255)		826 (328)	0.3
Flow-mediated dilatation, mm		0.27 (0.1)		0.26 (0.1)		0.28 (.01)		0.26 (0.1)		0.27 (0.1)	0.8
Males		0.28 (0.1)		0.25 (0.1)		0.28 (0.1)		0.31 (0.12)		0.23 (0.1)	0.7
Females		0.27 (0.09)		0.27 (0.09)		0.27 (0.1)		0.28 (0.08)		0.28 (0.1)	0.6
Flow-mediated dilatation, %		7.7 (3.3)		7.9 (3.4)		8.8 (4.0)		9.2 (3.2)		8.3 (4.5)	0.09
Males		7.2 (3.3)		6.2 (3)		7.5 (3.1)		8.2 (3.7)		5.8 (2.9)	0.8
Females		8.5 (3.3)		9.1 (3.1)		9.4 (4.2)		9.5 (9.3)		9.2 (4.7)	0.4
Distension, mm	**45**	0.09 (0.04)	**43**	0.08 (0.03)	**42**	0.08 (0.04)	**44**	0.08 (0.03)	**45**	0.09 (0.04)	0.7
Males	**28**	0.1 (0.05)	**17**	0.1 (0.04)	**10**	0.08 (0.03)	**13**	0.08 (0.03)	**12**	0.1 (0.04)	0.7
Females	**17**	0.09 (0.04)	**26**	0.07 (0.03)	**32**	0.08 (0.04)	**31**	0.08 (0.03)	**33**	0.09 (0.05)	0.6
Distension, %		12.3 (6.0)		11.4 (3.9)		12.5 (5.9)		11.5 (5.3)		13.7 (6.6)	0.4
Males		10.8 (4.4)		10.9 (3.4)		8.9 (3.9)		9.5 (4)		11.5 (4)	0.6
Females		15.1 (7.3)		11.7 (4.3)		13.6 (6)		12.4 (5.6)		14.5 (7.1)	0.8
Distension coefficient ($x10^{-3}.kPa^{-1}$)		9.0 (4.2)		8.4 (2.9)		9.1 (4.3)		8.5 (3.8)		10.5 (5.8)	0.2
Males		7.9 (3.2)		7.9 (2.4)		6.6 (2.9)		6.9 (2.9)		10.2 (8.1)	0.6
Females		11 (5)		8.7 (3.1)		10 (4.4)		10 (3.9)		10.6 (4.9)	0.8

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Right Common Carotid Artery											
Diameter, mm	**57**	6.6 (0.6)	**56**	6.5 (0.5)	**60**	6.4 (0.5)	**61**	6.5 (0.5)	**59**	6.4 (0.5)	***0.03***
Males	**37**	6.8 (0.6)	**21**	6.7 (0.6)	**17**	6.8 (0.3)	**18**	6.9 (0.4)	**17**	6.8 (0.4)	0.9
Females	**20**	6.3 (0.4	**35**	6.3 (0.4)	**43**	6.3 (0.3)	**43**	6.2 (0.5)	**42**	6.3 (0.4)	0.7
Distension, mm		0.7 (0.2)		0.7 (0.1)		0.8 (0.2)		0.7 (0.1)		0.7 (0.2)	0.5
Males		0.7 (0.2)		0.7 (0.2)		0.8 (0.2)		0.7 (0.2)		0.8 (0.1)	0.3
Females		0.7 (0.1)		0.8 (0.1)		0.7 (0.2)		0.7 (0.1)		0.7 (0.1)	0.8
Distension, %		10.7 (2.7)		11.5 (2.4)		11.8 (2.7)		11.1 (2.7)		11.7 (2.4)	0.1
Males		10.8 (3.1)		10.8 (3.6)		11.7 (2.9)		9.9 (2.8)		12.4 (2.6)	0.4
Females		10.7 (1.8)		11.9 (2.2)		11.8 (2.6)		11.6 (2.6)		11.4 (2.4)	0.9
Distension coefficient ($x10^{-3}.kPa^{-1}$)		32 .1 (0.7)		34.3 (6.5)		34.8 (8.1)		33.3 (7.5)		36.3 (13.4)	***0.05***
Males		30.2 (7.5)		30.7 (6)		30.5 (7.4)		28.2 (5.7)		37.8 (21.6)	0.1
Females		35.4 (5.7)		36.4 (5.8)		35.7 (8.3)		35.4 (7.2)		35.8 (8.3)	0.9
Intima-media thickness, mm		0.57 (0.1)		0.55 (0.07)		0.53 (0.05)		0.55 (0.07)		0.54 (0.09)	***0.04***
Males		0.58 (0.1)		0.56 (0.08)		0.52 (0.04)		0.56 (0.08)		0.57 (0.1)	0.8
Females		0.56 (0.08)		0.54 (0.06)		0.54 (0.07)		0.54 (0.06)		0.52 (0.06)	0.07
Carotid-radial PWV m/s		8.6 (1.1)		8.8 (1.6)		8.5 (1.2)		8.7 (1.3)		8.4 (1.2)	0.3
Males		8.6 (1.2)		8.6 (1.1)		8.9 (1.4)		9.1 (0.9)		8.9 (1)	0.2
Females		8.5 (0.8)		8.9 (1.8)		8.3 (1.1)		8.5 (1.3)		8.3 (1.2)	0.1

* Lowest quintile for dietary pattern score. ‡ *P* for trend across quintiles calculated with the dietary pattern score modelled on a continuous scale in general linear models. Data are mean, SD.

Table 8-5 Vascular Variables at Baseline According to Quintiles of Meat Based Dietary Pattern Score

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	*p*‡
Right Brachial Artery											
Diameter, mm	**62**	3.1 (0.5)	**63**	3.0 (0.5)	**65**	3.2 (0.6)	**63**	3.3 (0.6)	**62**	3.3 (0.6)	***<0.001***
Males	**14**	3.6 (0.6)	**17**	3.7 (0.4)	**25**	3.8 (0.4)	**26**	3.8 (0.4)	**36**	3.7 (0.4)	0.5
Females	**48**	2.9 (0.3)	**47**	2.8 (0.3)	**40**	2.8 (0.4)	**37**	3 (0.5)	**27**	2.8 (0.3)	0.8
Reactive hyperaemia, %		806 (236)		736 (221)		748 (361)		675 (173)		711 (180)	***0.02***
Males		657 (270)		550 (170)		726 (277)		642 (156)		614 (226)	0.9
Females		849 (209)		804 (198)		761 (402)		700 (183)		844 (265)	0.2
Flow-mediated dilatation, mm		0.31 (0.1)		0.25 (0.09)		0.27 (0.09)		0.25 (0.08)		0.29 (0.1)	0.8
Males		0.31 (0.1)		0.25 (0.06)		0.25 (0.08)		0.28 (0.1)		0.28 (0.1)	0.4
Females		0.29 (0.1)		0.26 (0.1)		0.26 (0.1)		0.26 (0.08)		0.3 (0.1)	0.9
Flow-mediated dilatation, %		9.4 (4.1)		8 (3.5)		8 (3.3)		7.9 (3.2)		8.7 (4.2)	0.3
Males		8.8 (4)		5.1 (2)		6.4 (2.3)		7.2 (3.3)		7.4 (3.6)	0.7
Females		9.6 (4.2)		9 (3.4)		9 (3.5)		8.4 (3.1)		10.3 (4.5)	1.0
Distension, mm	**42**	0.08 (0.04)	**52**	0.09 (0.04)	**39**	0.09 (0.04)	**32**	0.09 (0.04)	**44**	0.08 (0.04)	1.0
Males	**9**	0.09 (0.04)	**14**	0.1 (0.04)	**15**	0.09 (0.05)	**17**	0.1 (0.04)	**25**	0.09 (0.03)	0.8
Females	**33**	0.08 (0.08)	**38**	0.09 (0.04)	**24**	0.09 (0.03)	**25**	0.08 (0.02)	**19**	0.08 (0.04)	0.6
Distension, %		12.2 (6.2)		13.2 (6.1)		13.3 (6.3)		11.8 (4.4)		11 (4.6)	0.1
Males		10 (4.7)		11 (3.1)		10 (4.5)		11.1 (5.4)		10.1 (2.9)	0.8
Females		12.7 (6.5)		13.9 (6.8)		15.4 (6.4)		12.3 (3.7)		12.3 (6)	0.6
Distension coefficient ($x10^{-3}.kPa^{-1}$)		9.5 (6.0)		9.7 (4.3)		9,6 (4.4)		8.6 (8.2)		8.1 (3.4)	***0.05***
Males		9.7 (9.8)		8.1 (2.2)		7.3 (3.2)		8.1 (3.9)		7.3 (2.2)	0.2
Females		9.4 (4.7)		10.2 (4.7)		11.1 (4.5)		9 (2.7)		9.1 (4.4)	0.6

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	*p*‡
Right Common Carotid Artery											
Diameter, mm	**56**	6.4 (0.5)	**62**	6.5 (0.5)	**58**	6.5 (0.5)	**57**	6.5 (0.6)	**60**	6.5 (0.5)	0.4
Males	**14**	6.6 (0.6)	**16**	7 (0.5)	**23**	6.8 (0.4)	**23**	6.9 (0.5)	**34**	6.7 (0.4)	0.6
Females	**42**	6.3 (0.4)	**46**	6.3 (0.4)	**35**	6.3 (0.4)	**34**	6.2 (0.4)	**26**	6.2 (0.5)	0.2
Distension, mm		0.7 (0.2)		0.7 (0.1)		0.7 (0.1)		0.7 (0.2)		0.8 (0.2)	0.5
Males		0.7 (0.2)		0.8 (0.2)		0.7 (0.2)		0.8 (0.2)		0.8 (0.2)	0.2
Females		0.7 (0.2)		0.7 (0.1)		0.7 (0.1)		0.7 (0.2)		0.7 (0.1)	0.5
Distension, %		11.5 (2.9)		11.5 (2.5)		10.7 (2.4)		11.4 (2.4)		11.6 (2.8)	0.2
Males		10.6 (3.1)		10.8 (2.6)		10.3 (2.8)		11.5 (2.3)		11.4 (3.3)	0.2
Females		11.8 (2.8)		11.7 (2.4)		10.9 (2)		11.4 (2.5)		11.8 (1.9)	0.4
Distension coefficient ($x10^{-3}.kPa^{-1}$)		36.5 (13.8)		34.8 (6.8)		32.6 (7.6)		33.1 (7.5)		34.0 (7.4)	0.07
Males		35 (4.8)		30.7 (5.4)		29.7 (7)		31.4 (6.3)		31.9 (7.7)	0.7
Females		37 (7.6)		36.2 (6.7)		34.6 (7.5)		34.2 (8.1)		36.7 (6.1)	0.3
Intima-media thickness, mm		0.54 (0.07)		0.55 (0.07)		0.55 (0.08)		0.55 (0.09)		0.55 (0.08)	0.5
Males		0.57 (0.1)		0.57 (0.07)		0.55 (0.09)		0.55 (0.1)		0.57 (0.09)	0.8
Females		0.53 (0.06)		0.54 (0.07)		0.54 (0.07)		0.54 (0.08)		0.52 (0.06)	0.8
Carotid-radial PWV m/s		8.3 (1.2)		8.6 (1.2)		9.0 (1.6)		8.5 (1.2)		8.6 (1.0)	0.4
Males		9 (1.4)		9.1 (1.1)		8.8 (1.3)		8.6 (1.2)		8.7 (1)	0.2
Females		8.2 (1.1)		8.3 (1.2)		9 (1.8)		8.5 (1.2)		8.5 (1.1)	0.2

* Lowest quintile for dietary pattern score. ‡ *P* for trend across quintiles calculated with the dietary pattern score modelled on a continuous scale in general linear models. Data are mean, SD.

Table 8-6 Vascular Variables at Baseline According to Quintiles of Snack Dietary Pattern Score

	n	*Q1*	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Right Brachial Artery											
Diameter, mm	**61**	3.1 (0.5)	**63**	3.1 (0.6)	**64**	3.4 (0.6)	**65**	3.1 (0.5)	**64**	3.3 (0.6)	0.3
Males	**19**	3.7 (0.3)	**20**	4.0 (0.3)	**36**	3.8 (0.4)	**21**	3.6 (0.5)	**22**	3.8 (0.5)	0.9
Females	**42**	2.9 (0.2)	**43**	2.8 (0.4)	**28**	2.8 (0.3)	**44**	2.8 (0.3)	**42**	3 (0.4)	0.2
Reactive hyperaemia, %		770 (257)		721 (332)		721 (228)		739 (256)		729 (242)	0.6
Males		643 (188)		624 (289)		660 (227)		624 (231)		620 (200)	0.8
Females		827 (265)		765 (344)		792 (210)		793 (252)		790 (244)	0.7
Flow-mediated dilatation, mm		0.27 (0.1)		0.26 (0.1)		0.27 (0.1)		0.28 (0.1)		0.28 (0.1)	0.4
Males		0.23 (0.1)		0.25 (0.09)		0.27 (0.1)		0.3 (0.01)		0.3 (0.1)	***0.03***
Females		0.29 (0.09)		0.27 (0.09)		0.26 (0.08)		0.26 (0.1)		0.28 (0.1)	0.6
Flow-mediated dilatation, %		8.5 (3.7)		8.4 (3.7)		7.9 (3.3)		8.7 (4.1)		8.4 (3.9)	0.8
Males		5.9 (2.9)		6.4 (2.9)		7 (3.2)		8.3 (3.9)		7.1 (3.1)	0.06
Females		9.6 (3.4)		9.3 (3.7)		9 (3.2)		8.9 (4.2)		9.1 (4.1)	0.5
Distension, mm	**45**	0.07 (0.03)	**40**	0.09 (0.04)	**49**	0.09 (0.04)	**38**	0.09 (0.04)	**47**	0.09 (0.04)	***0.05***
Males	**19**	0.1 (0.04)	**11**	0.1 (0.05)	**27**	0.09 (0.05)	**12**	0.1 (0.04)	**11**	0.09 (0.03)	0.3
Females	**26**	0.07 (0.02)	**29**	0.08 (0.03)	**22**	0.09 (0.03)	**26**	0.09 (0.05)	**36**	0.1 (0.05)	***0.002***
Distension, %		10.6 (3.9)		12.5 (4.3)		12.2 (6.1)		13.7 (6.7)		12.7 (6.3)	0.07
Males		10 (3.1)		12.1 (4.4)		10.2 (4.6)		1.5 (3.9)		9.4 (3.3)	0.3
Females		10.8 (4.1)		12.7 (4.4)		14.8 (6.8)		14.6 (7.5)		14.9 (6.9)	***0.003***
Distension coefficient ($x10^{-3}.kPa^{-1}$)		7.8 (2.8)		9.7 (5.2)		8.9 (4.3)		10.0 (4.7)		9.3 (4.4)	0.2
Males		7.4 (2.4)		10.6 (8.2)		7.4 (3.2)		8.4 (2.9)		6.9 (2.3)	0.2
Females		8 (2.9)		9.3 (3.4)		10.7 (4.7)		10.6 (5.2)		10.9 (4.8)	***0.003***

	n	*Q1*	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Right Common Carotid Artery											
Diameter, mm	**58**	6.4 (0.47)	**58**	6.4 (0.45)	**61**	6.6 (0.50)	**61**	6.4 (0.54)	**55**	6.6 (0.57)	0.3
Males	**16**	6.8 (0.4)	**18**	6.6 (0.4)	**36**	6.8 (0.5)	**19**	6.8 (0.4)	**21**	6.9 (0.6)	0.2
Females	**42**	6.3 (0.4)	**40**	6.3 (0.4)	**25**	6.3 (0.3)	**42**	6.2 (0.5)	**34**	6.3 (0.4)	0.8
Distension, mm		0.8 (0.2)		0.8 (0.2)		0.7 (0.2)		0.7 (0.1)		0.7 (0.2)	***0.05***
Males		0.8 (0.2)		0.8 (0.2)		0.7 (0.2)		0.7 (0.2)		0.7 (0.2)	0.1
Females		0.7 (0.2)		0.8 (0.1)		0.7 (0.1)		0.7 (0.1)		0.7 (0.1)	0.2
Distension, %		11.7 (2.8)		11.8 (2.8)		11.0 (2.5)		11.6 (2.6)		10.6 (2.2)	0.08
Males		12.1 (3.2)		11.5 (2.5)		10.8 (2.5)		11 (2.6)		10.2 (2.4)	0.1
Females		11.5 (2.6)		12 (2.1)		11.4 (2.5)		11.8 (2.6)		10.8 (2)	0.5
Distension coefficient ($x10^{-3}.kPa^{-1}$)		34.6 (7.4)		36.5 (13.1)		32.8 (7.4)		34.8 (8.0)		32.1 (7.1)	0.09
Males		32.7 (7.2)		35.8 (22.2)		30.7 (5.8)		31 (6.8)		29 (6.7)	0.1
Females		35.3 (7.4)		36.8 (6.1)		36 (8.4)		36.5 (8.1)		34.1 (6.6)	0.6
Intima-media thickness, mm		0.55 (0.09)		0.54 (0.07)		0.54 (0.07)		0.53 (0.07)		0.55 (0.59)	0.2
Males		0.59 (0.1)		0.54 (0.08)		0.56 (0.08)		0.54 (0.05)		0.6 (0.01)	0.9
Females		0.52 (0.07)		0.54 (0.07)		0.53 (0.06)		0.53 (0.08)		0.056 (0.06)	0.2
Carotid-radial PWV m/s		8.6 (1.0)		8.6 (1.7)		8.5 (1.1)		8.5 (1.1)		8.7 (1.4)	0.8
Males		8.6 (1.1)		9.3 (1.4)		8.7 (1.2)		8.5 (0.8)		8.9 (1.3)	0.9
Females		8.6 (1)		8.3 (1.7)		8.3 (1)		8.5 (1.2)		8.6 (1.5)	0.7

* Lowest quintile for dietary pattern score. ‡ *P* for trend across quintiles calculated with the dietary pattern score modelled on a continuous scale in GLM. Data are mean, SD.

Table 8-7 Regression coefficient (95% CI) for vascular variables according to quintiles of dietary pattern score[1]

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Right Common Carotid Artery									
IMT (mm)									
Health Conscious									
Model 1* **n = 307**	-0.03 (-0.06, 0.002)	0.07	-0.04 (-0.07, -0.01)	0.004	-0.03 (-0.06, 0.001)	0.06	-0.04 (-0.07, -0.1)	***0.007***	***0.02***
Males	0.003 (-0.06, 0.06)	0.9	-0.04 (-0.1, 0.02)	0.2	-0.01 (-0.05, 0.07)	0.8	-0.02 (-0.08, 0.05)	0.6	0.7
Females	-0.05 (-0.1, -0.003)	0.04	-0.05 (-0.1, -0.009)	0.02	-0.04 (-0.09, 0)	0.05	-0.07 (-0.1, -0.03)	***0.002***	***0.02***
Model 2† **n = 271**	-0.02 (-0.05, 0.02)	0.4	-0.03 (-0.06, 0.006)	0.1	-0.009 (-0.04, 0.03)	0.6	-0.02 (-0.06, 0.02)	0.3	0.6
Males	0.002 (-0.06, 0.06)	1.0	-0.04 (-0.1, 0.03)	0.3	0.02 (-0.05, 0.08)	0.6	0.005 (-0.06, 0.07)	0.9	0.9
Females	-0.03 (-0.08, 0.02)	0.2	-0.04 (-0.08, 0.007)	0.1	-0.03 (-0.07, 0.01)	0.2	-0.04 (-0.09, 0)	***0.05***	***0.05***
Meat Based									
Model 1*	0.009 (-0.02, 0.04)	0.6	0.008 (-0.02, 0.03)	0.6	0.009 (-0.21, 0.04)	0.5	0.003 (-0.03, 0.03)	0.8	0.9
Model 2†	0.001 (-0.03, 0.03)	0.9	0.004 (-0.03, 0.3)	0.8	-0.002 (-0.03, 0.03)	0.9	-0.003 (-0.03, 0.03)	0.8	0.9
Snack									
Model 1*	-0.007 (-0.04, 0.02)	0.7	-0.01 (-0.04, 0.02)	0.5	-0.01 (-0.04, 0.02)	0.4	-0.02 (-0.1, 0.05)	0.2	0.2
Model 2†	-0.01 (-0.04, 0.02)	0.5	-0.03 (=-.-6, 0.002)	0.07	-0.02 (-0.05, 0.009)	0.2	-0.01 (-0.02, 0.04)	0.5	0.8
Brachial Artery									
Distension (mm)									
Health Conscious									
Model 1*	-0.008 (-0.02, 0.009)	0.4	-0.005 (-0.02, 0.01)	0.6	-0.01 (-0.03, 0.002)	0.09	0.004 (-0.01, 0.02)	0.7	0.9
Model 2†	-0.02 (-0.03, 0.002)	0.09	-0.007 (-0.03, 0.01)	0.4	-0.02 (-0.04, -0.002)	0.03	0 (-0.02, 0.02)	1.0	0.8
Meat Based									
Model 1*	-0.01 (-0.006, 0.03)	0.2	0.008 (-0.009,0.03)	0.4	0.002 (-0.02, 0.02)	0.8	-0.001 (-0.02, 0.02)	0.9	0.6
Model 2†	0.009 (-0.007, 0.03)	0.3	0.008 (-0.01, 0.03)	0.4	0.001 (-0.02, 0.02)	0.9	-0.005 (-0.23, 0.01)	0.6	0.4

Snack									
Model 1*	0.02 (0, 0.03)	0.06	0.02 (0, 0.03)	0.05	0.02 (-0.002, 0.03)	0.09	0.02(-0.001, 0.03)	0.07	0.1
Males	0.01 (-0.02, 0.05)	0.5	-0.003 (-0.04, 0.03)	0.9	-0.005 (-0.04, 0.03)	0.8	-0.01 (-0.05, 0.02)	0.5	0.2
Females	0.02 (-0.001, 0.04)	0.06	0.03 (0.005, 0.05)	0.02	0.02 (0.003, 0.04)	0.03	0.03 (0/01, 0.05)	***0.003***	***0.003***
Model 2†	0.02 (-0.002, 0.03)	0.09	0.02 (0.001, 0.04)	0.04	0.02 (-0.003, 0.03)	0.1	0.02 (0, 0.04)	***0.05***	0.1
Males	0.006 (-0.03, 0.44)	0.8	-0.004 (-0.04, 0.03)	0.8	-0.005 (-0.04, 0.03)	0.8	-0.02 (-0.06, 0.1)	0.2	0.1
Females	0.01 (-0.005, 0.03)	0.2	0.03 (0.006, 0.05)	0.1	0.02 (-0.003, 0.04)	0.1	0.03 (0.006, 0.05)	0.0	***0.02***

[1]For tables 8.7 – 8.11 quintile 1 is the lowest for dietary pattern score (referent set at 0). [2]Beta coefficient values are calculated as highest minus lowest quintiles based on general linear models. [3] *p* for trend across quintiles calculated in GLM with dietary pattern modelled continuously (score). *Adjusted for baseline arterial diameter, skin and room temperature † Adjusted additionally for age, sex, social class, physical activity, LDL cholesterol, triglycerides, WHR, mean arterial blood pressure, fasting insulin and energy intake.

Table 8-8 Regression coefficients (95% CI) for CVD risk factors (obesity) according to quintiles of dietary pattern score[1]

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Body Mass Index									
Health conscious									
Model 1[*]: **n= 321**	-1.6 (-3, -0.2)	0.02	-1.4 (-2.8, -0.1)	0.04	-1.1 (-2.5, 0.3)	0.1	-0.8 (-2.2, 0.5)	0.2	0.5
Model 2 [†] **n= 213**	-0.5 (-1.7, 0.8)	0.5	-0.5 (-1.7, 0.8)	0.5	-0.09, (-1.3, 1.2)	0.9	-0.8 (-0.5, 2.2)	0.2	0.2
Meat based									
Model 1[*]	-0.5 (-0.8, 1.9)	0.4	-1 (-0.4, 2.3)	0.2	-0.6 (0.8, 2.0)	0.4	-0.5 (-1.9, 0.9)	0.5	0.6
Model 2 [†]	0.5 (-0.7, 1.7)	0.4	0.5 (-0.7, 1.7)	0.4	0.09 (-1.1, 1.3)	0.9	-0.7 (-1.9, 0.5)	0.3	0.2
Snack									
Model 1[*]	0.4 (-1, 1.7)	0.6	0.8 (-0.5, 2.2)	0.2	1.6 (0.2, 2.9)	0.03	1.1 (-0.2, 2.5)	0.1	***0.03***
Model 2 [†]	0.8 (-1.1, 1.4)	0.8	0.2 (-1.1, 1.4)	0.8	0.9 (-0.3, 2.2)	0.1	0.3 (-0.9, 1.6)	0.6	0.5
Waist circumference, cm									
Health conscious									
Model 1[*]	-4.8 (-8.8,-1.4)	0.006	-6.5 (-10,-3)	<0.001	-5.8 (-9.3,-2.4)	0.001	-5.7 (-9.1,-2.2)	***0.001***	***0.002***
Model 2 [†]	-2.9 (-6.0, 0.3)	0.07	-3.5 (-6.7,-0.3)	0.03	-2.7 (-5.8, 0.5)	0.1	-2.9 (-6.2,0.3)	0.08	0.2
Meat based									
Model 1[*]	1.3 (-2.2, 4.8)	0.5	3.8 (0.3, 7.3)	0.03	2.3 (-1.2, 5.8)	0.2	1.9 (-1.7, 5.4)	0.3	0.2
Model 2 [†]	0.2 (-2.6, 3.1)	0.9	1.5 (-1.3, 4.4)	0.3	-0.2 (-3.0, 2.6)	0.9	-1.5 (-4.4, 1.4)	0.1	0.3
Snack									
Model 1[*]	-1 (-3.6, 3.4)	0.9	3.7 (0.2,7.21)	0.04	2.4 (-1.1,5.9)	0.2	3.2 (-0.3,6.7)	0.07	***0.03***
Model 2 [†]	0.7 (-2.1, 3.6)	0.6	-0.3 (-2.6, 3.3)	0.9	-1.1 (-1.7, 3.9)	0.5	1.0 (-1.9, 4.0)	0.5	0.5
Waist:Hip ratio									
Health conscious									
Model 1[*]	-0.04 (-0.06, 0.02)	0.001	-0.06 (-0.08, 0.04)	<0.001	-0.05 (-0.07, 0.03)	<0.001	-0.05 (-0.08, -0.03)	***<0.001***	***<0.001***

Model †	-0.02 (-0.04, 0.00)	0.06	-0.03 (-0.05, 0.01)	0.003	-0.02 (-0.04, -0.002)	0.03	-0.02(-0.04,0.002)	0.08	0.1
Meat Based									
Model 1*	0.004 (-0.2, 0.3)	0.7	0.03 (0.009 ,0.06)	0.008	0.02 (-0.005,0.04)	0.1	0.03 (0.009,0.06)	***0.007***	***0.003***
Model †	0.003 (-0.02, 0.2)	0.7	0.02 (-0.002, 0.03)	0.08	-0.00 (-0.02,0.02)	1	0 004 (-0.01.0.02)	0.7	0.8
Snack									
Model 1*	-0.003 (-0.3, 0.2)	0.8	0.03 (0.008, 0.06)	0.01	0.02 (-0.009,0.04)	0.2	0.02 (-0.002,0.05)	0.07	***0.02***
Model 2†	0.00 (-0.02, 0.02)	1	-0.002 (-0.02, 0.02)	0.8	0.008 (-0.01,0.03)	0.4	0.009 (-0.009,0.03)	0.4	0.2

*Unadjusted,† Adjusted for age, sex and energy intake.

Table 8-9 Regression coefficients (95% CI) for CVD risk factors (blood pressure) according to quintiles of dietary pattern score1

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Systolic (mm Hg)									
Health conscious									
Model 1[*] **:n=321**	-2.2 (-5.8, 1.5)	0.2	-3.9 (-7.6, -0.25)	0.04	-2.8 (-6.5, 0.87)	0.1	-7.4 (-11.1, -3.7)	***<0.001***	***<0.001***
Model 2[†] **:n=310**	1 (-2.3, 4.4)	0.5	-0.08 (-3.5,3.3)	1	1.4 (-2, 4.8)	0.4	-3.6 (7.1, -0.1)	0.05	0.06
Meat Based									
Model 1[*]	2.5 (-1.1, 6.2)	0.17	3.8 (0.15, 7.4)	0.04	6.5 (2.8, 10.1)	0.001	6.9 (3.2, 10.5)	***<0.001***	***<0.001***
Model 2[†]	1.2 (-2.1,4.5)	0.5	1.4 (-1.9, 4.7)	0.4	4.6 (1.3, 7.9)	0.007	5.5 (2.1, 8.8)	***0.001***	***<0.001***
Snack									
Model 1[*]	-2.3 (-6, 1.5)	0.2	1.7 (-2, 5.4)	0.4	2 (-1.7, 5.7)	0.3	2 (-1.7, 5.7)	0.3	0.06
Model 2[*†]	-2.4 (-5.8, 8.9)	0.2	-1 (-4.4, 2.4)	0.6	0.4 (-2.9, 3.8)	0.8	0.7 (-2.8, 4.1)	0.7	0.2
Diastolic (mm Hg)									
Health conscious									
Model 1[*]	-1.6 (-4.2, 1.1)	0.2	-3.8 (-6.4, -1.1)	0.005	-1.8 (-6.4, -1.1)	0.2	-6.3 (-9, -3.7)	***<0.001***	***<0.001***
Model 2[†]	0.4 (-2.1, 2.9)	0.7	-1.7 (-4.2, 0.8)	0.2	0.4 (-2.1, 3)	0.7	-4.6 (-7.3, 1.9)	***0.001***	***0.002***
Meat Based									
Model 1[*]	0.96 (-1.7, 3.6)	0.06	2.6 (-0.1, 5.3)	0.06	2.4 (-0.3, 5.1)	0.08	3.7 (1.0, 6.4) [3]	***0.008***	***0.004***
Model 2[*†]	0.2 (-2.4, 2.7)	0.9	1.2 9-1.3, 3.7)	0.4	1.5 (-1.1, 4)	0.3	3.5 (0.9, 6)	***0.008***	***0.003***
Snack									
Model 1[*]	-1 (-3.7, 1.7)	0.5	2 (-0.7, 4.7)	0.2	2.1 (-0.6, 4.8)	0.1	2 (-0.7, 4.7)	0.1	***0.02***
Model 2[*†]	-1.2 (-3.7, 1.4)	0.4	0.5 (-2.1, 3.1)	0.7	1 (-1.6, 3.6)	0.4	0.7 (-1.9, 3.3)	0.6	0.1
Mean Arterial Pressure (mm Hg)									
Health conscious									

Model 1*	-2.3 (-5.3, 0.78)	0.1	-4.1 (-7.1, -1.1)	0.008	-2.7 (-5.7, 0.3)	0.08	-6.8 (-9.9, -3.7)	***<0.001***	***<0.001***
Model 2†	0.1 (-2.8, 3)	0.9	-1.5 (-4.4, 1.5)	0.3	0.3 (-2.6, 3.3)	0.8	-4.1 (-7.2, 2.1)	***0.009***	***0.02***
Meat Based									
Model 1*	1.6 (-1.4, 4.7)	0.3	2.8 (-0.3, 5.9)	0.08	0.02 (-3.2, 2.6)	0.01	4.9 (1.8, 7.9)	***0.002***	***0.004***
Model 2†	0.8 (-2.1,3.7)	0.6	0.8 (-2.2, 5.5)	0.07	2.6 (-0.2, 5.5)	0.07	3.7 (0.8, 6.6)	***0.01***	***0.002***
Snack									
Model 1*	-1.6 (-5, 1.5)	0.3	1.6 (-1.5, 4.7)	0.3	1.8 (-1.2, 4.9)	0.2	2.2 (-0.8, 5.3)	0.2	0.5
Model 2†	-2 (-4.9, 0.9)	0.2	-0.6 (-3.5, 2.4)	0.7	0.5 (-2.4, 3.4)	0.7	0.9 (-2.1, 3.9)	0.5	0.1
Heart Rate (beats per minute)									
Health conscious									
Model 1*	-1 (-4.5, 2.6)	0.6	-2.1 (-5.6, 1.5)	0.3	0.4 (-3.2, 4)	0.8	-5.7 (-9.3, -2.1)	***0.002***	***0.02***
Model 2†	-0.6 (-4.3, 2.9)	0.7	-2.2 (-5.9, 1.4)	0.2	0.2 (-3.4, 3.8)	0.9	-5.3 (-9.2, 1.5)	***0.007***	***0.02***
Meat Based									
Model 1*	2.6 (1, 6.3)	0.2	3.4 (-0.2, 2.7)	0.06	4.2 (0.6, 7.8)	0.03	2.6 (-1.04, 6.2)	0.2	0.1
Model 2†	3 (-0.6)	0.1	3.8 (0.2,7.4)	0.04	4.5 (1, 8.1)	0.01	3.6 (0.03, 7.3)	0.05	0.7
Snack									
Model 1*	0.9 (-2.7, 4.5)	0.6	1.3 (-2.3, 5)	0.5	2.9 (-0.7, 6.5)	0.1	5.1 (1.5, 8.7)	***0.006***	***0.003***
Model 2†	0.3 (-3.3, 3.9)	0.9	2 (-1.7, 5.6)	0.3	1.4 (-2.2, 5.1)	0.4	3.2 (-0.5, 6.9)	0.09	***0.04***

*Unadjusted,† Adjusted for age, sex, height, WHR, physical activity, social class, smoking practice, LDL cholesterol, triglycerides and energy intake.

Table 8-10: Regression coefficients (95% CI) for CVD risk factors (fasting serum lipid concentrations) and dietary pattern quintiles

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Total-cholesterol									
Health conscious									
Model 1[*] **:n= 319**	-0.4 (-0.7, -1)	0.01	-0.3 (-0.6, -0.06)	0.02	-0.2 (-0.5, 0.04)	0.09	-0.5 (-0.8, -0.2)	***0.002***	***0.01***
Model 2[†] **n = 313**	-0.3 (-0.6, 0.03)	0.04	-0.3 (-0.6, 0.04)	0.08	-0.1 (-0.4, 0.2)	0.3	-0.3 (-0.7, 0.006)	0.02	0.1
Meat Based									
Model 1[*]	-0.2 (-0.5, 0.08)	0.2	-0.07 (-0.4, 0.2)	0.7	-0.1 (-0.4, 0.2)	0.5	-0.09 (-0.4, 0.2)	0.5	0.7
Model 2[†]	-0.3 (-0.6, 0.03)	0.08	-0.2 (-0.5, 0.1)	0.2	-0.2 (-0.5, 0.1)	0.3	-0.2 (-0.5, 0.1)	0.2	0.5
Snack									
Model 1[*]	0.05 (-0.2,0.3)	0.7	0.1 (-0.2, 0.4)	0.4	0.07 (-0.2,0.4)	0.6	0.3 (0.01, 0.6)	0.04	0.1
Model 2[†]	0.01 (-0.3, 0.3)	0.9	-0.07 (-0.4, 0.2)	0.7	0.05 (-0.3, 0.3)	0.8	0.1 (-0.2, 0.4)	0.5	0.8
LDL-cholesterol									
Health conscious									
Model 1[*]	-0.3 (-0.61, -0.07)	0.02	-0.4 (-9.63, -0.1)	0.008	-0.3 (-0.6, 0.01)	0.06	-0.4 (-0.67. -0.16)	***0.002***	***0.01***
Model 2[†]	-0.2 (-0.2,0.07)	0.1	-0.2 (-0.5,0.02)	0.07-	0.09 (-0.4,0.2)	0.5	-0.3 (-0.6,-0.05)	0.02	0.2
Meat Based									
Model 1[*]	-0.1 (-0.4, 0.2)	0.5	0.05 (-0.2, 0.3)	0.7	-0.02 (-0.3,0.2)	0.9	-0.07 (-0.3, 0.2)	0.6	0.8
Model 2[†]	-0.2 (-0.4, 0.1)	0.3	-0.08 (-0.3, 0.2)	0.6	-0.1 (-0.4, 0.2)	0.4	-0.2 (0.5, 0.09)	0.2	0.4
Snack									
Model 1[*]	0.09 (-0.2, 0.4)	0.5	0.2 (-0.03, 0.5)	0.09	0.1 (-0.1, 0.4)	0.4	0.3 (0.05, 0.6)	***0.02***	***0.03***
Model 2[†]	0.04 (-0.2, 0.3)	0.8	0.03 (-0.2, 0.3)	0.8	0.05 (-0.2,0.3)	0.7	0.2 (-0.1, 0.5)	0.2	0.3
HDL-cholesterol									
Health Conscious									
Model 1[*]	0.1 (-0.01, 0.2)	0.08	0.1 (-0.01, 0.2)	0.08	0.1 (0.01, 0.3)	0.03	0.1 (-0.009, 0.2)	0.07	0.08
Model 2[†]	0.03 (-0.08, 0.1)	0.6	0.02 (-0.1, 0.1)	0.7	0.04 (-0.08, 0.2)	0.6	0.06 (-0.08, 0.2)	0.4	0.6
Meat Based									

	Q2	P^2	*Q3*	P^2	*Q4*	P^2	*Q5*	P^2	p‡
Model 1*	-0.08 (-0.2, 0.05)	0.2	-0.1 (-0.2, 0.005)	0.04	-0.1 (-0.2, 0.01)	0.07	-0.04 (-0.2, 0.08)	0.5	0.4
Model 5†	-0.05 (-0.2, 0.06)	0.4	-0.05 (-0.2, 0.07)	0.4	-0.05 (-0.2, 0.06)	0.4	0.05 (-0.07, 0.2)	0.4	0.8
Snack									
Model 1*	0.07 (-0.05, 0.19)	0.27	-0.11 (-0.2, 0.01)	0.07	-0.05 (0.2, 0.07)	0.38	-0.08 (-0.2, 0.05)	0.2	***0.05***
Model 2†	0.09 (-0.02, 0.2)	0.1	-0.04 (-0.2, 0.08)	0.5	-0.02 (-0.1, 0.1)	0.7	-0.03 (-0.2, 0.10	0.7	0.1
VLDL-cholesterol									
Health conscious									
Model 1*	-0.09 (-0.2, 0.01)	0.06	-0.1 (0.2, 0.001)	0.02	-0.1 (-0.2, -0.04)	0.009	-0.2 (-0.2,-0.6)	***<0.001***	***<0.001***
Model 2†	-0.07 (0.2, 0.03)	0.1	-0.05 (-0.1, 0.05)	0.2	-0.1 (-0.2, 0.004)	0.05	-0.1 (-0.2, -0.001)	0.02	0.06
Meat Based									
Model 1*	0.04 (-0.06, 0.1)	0.08	0.01 (-0.08, 0.1)	0.06	0.04 (-0.06, 0.1)	0.09	0.03 (-0.07, 0.1)	0.3	0.7
Model 2†	0.03 (-0.07, 0.1)	0.6	-0.04 (-0.1, 0.05)	0.4	-0.004 (-0.1, 0.09)	0.9	-0.03 (-0.1, 0.06)	0.5	0.6
Snack									
Model 1*	-0.03 (-0.1, 0.07)	0.6	0.008 (-0.09, 0.1)	0.9	0 (-0.1, 0.1)	1	0.06 (-0.04, 0.2)	0.2	0.06
Model 2†	-0.04 (-0.1, 0.05)	0.4	-0.06 (-0.2, 0.04)	0.2	-0.07 (-0.2, 0.03)	0.2	-0.03 (-0.1, 0.07)	0.5	0.5
Triacylglycerol									
Health conscious									
Model 1*	-0.2 (-0.5, -0.02)	0.08	-0.2 (-0.4, 0.005)	0.06	-0.3 (-0.6, -0.8)	0.01	-0.3 (-0.6, -0.1)	***0.002***	***<0.001***
Model 2†	-0.1 (-0.3, 0.06)	0.1	-0.1 (-0.3, 0.1)	0.2	-0.2 (-0.4, -0.007)	0.05	-0.2 (-0.5, 0)	0.02	0.06
Meat Based									
Model 1*	0.09 (-0.2, 0.3)	0.3	0.03 (-0.2, 0.2)	0.5	0.08 (-0.1, 0.3)	0.3	0.06 (-0.2, 0.3)	0.3	0.6
Model 2†	0.06 (-0.1, 0.3)	0.5	-0.1 (-0.3, 0.1)	0.6	-0.009 (-0.2, 0.2)	0.7	-0.07 (-0.3, 0.1)	0.4	0.6
Snack									
Model 1*	-0.07 (-0.3, 0.1)	0.5	0.02 (-0.2, 0.2)	0.9	0.002 (-0.2, 0.2)	1	0.1 (-0.08, 0.3)	0.2	0.2
Model 2†	-0.09 (-0.2, 0.1)	0.2	-0.1 (-0.3, 0.09)	0.2	-0.1 (-0.4, 0.06)	0.2	-0.08 (-0.3, 0.1)	0.5	0.6

*Unadjusted. † Adjusted for age, sex, WHR, physical activity, social class, smoking practice, mean arterial blood pressure, and energy intake. All measurements based on fasting concentrations and in mmoL/L unless stated.

Table 8-11: Regression coefficients (95% CI) for insulin, insulin resistance and C-reactive protein and dietary pattern quintiles

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Insulin (pmol/L)									
Health Conscious									
Model 1[*] **n= 319**	-10.1 (-19.3, -0.8)	0.03	-5.2 (-14.6, 4.1)	0.3	-10.4 (-19.7, -1.1)	0.3	-10.4 (-19.7, -1)	***0.03***	***0.04***
Model 2[†] **n = 313**	-7.2 (-14.7, 0.3)	0.06	-4.4 (-12.2, 3.3)	0.3	-8.8 (-16, -1.1)	0.03	-8.5 (-17, 0.4)	0.05	0.1
Meat Based									
Model 1[*]	-2.4 (-11.8, 7)	0.6	5.2 (-4.2, 14.5)	0.3	4.7 (-4.7, 14.1)	0.3	-2.8 (-12.1, 6.6)	0.6	0.9
Model 2[†]	-0.01 (-1.2, 1.2)	1	0.8 (-0.5, 2)	0.2	1 (-0.3, 2.2)	0.1	-0.08 (-1.4, 1.3)	0.9	0.3
Snack									
Model 1[*]	1.5 (-7.8, 10.9)	0.7	0.5 (-8.8, 9.8)	0.9	12.6 (3.2, 21.9)	0.008	6.9 (-2.4, 16.3)	0.1	***0.02***
Model 2[†]	0.3 (-1, 1.5)	0.7	-0.7 (-2, 0.6)	0.3	1.1 (-0.2, 2.3)	0.1	0.2 (-1.2, 1.5)	0.8	0.2
HOMA									
Health Conscious									
Model 1[*]	-0.5 (-0.9, -0.04)	0.03	-0.3 (-0.7, 0.2)	0.05	-0.5 (-0.9, -0.07)	0.02	-0.5 (-0.9, -0.08)	***0.02***	***0.03***
Model 2[†]	-0.5 (-0.9, -0.04)	0.03	-0.3 (-0.7, 0.2)	0.2	-0.5 (-0.9, -0.07)	0.02	-0.5 (-0.9, -0.08)	0.02	0.3
Meat Based									
Model 1[*]	-0.1 (-0.6, 0.3)	0.5	0.2 (-0.2, 0.7)	0.3	0.2 (-0.2, 0.6)	0.4	-0.1 (-0.5, 0.3)	0.6	0.8
Model 2[†]	0.01 (-0.3, 0.3)	0.9	0.2 (-0.2, 0.6)	0.2	0.2 (-0.1, 0.6)	0.2	0.006 (-0.4, 0.4)	0.4	0.4
Snack									
Model 1[*]	0.06 (-0.2, 0.3)	0.6	0.003 (-0.2, 0.2)	1	0.4 (0.2, 0.6)	0.001	0.2 (0.06, 0.5)	***0.01***	***0.03***
Model 2[†]	-0.04 (-0.3, 0.4)	0.8	-0.2 (-0.5, 0.2)	0.3	0.3 (-0.05, 0.6)	0.09	0.006 (-0.4, 0.4)	0.9	0.3
C-reactive protein									
Health Conscious									
Model 1[*]	-1.1 (-2.3, 0.1)	0.07	-0.003 (-1.2, 1.2)	1.0	0.1 (-1.1, 1.3)	0.8	-0.5 (-1.7, 0.7)	0.4	0.8

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Model 2†	-0.8 (-2.0, 0.5)	0.2	0.3 (-0.9, 1.6)	1.6	-0.3 (-0.9, 1.6)	0.6	0.03 (-1.3, 1.4)	1.0	0.4
Meat Based									
Model 1*	1.0 (-0.2, 2.2)	1.0	0.8 (-0.4, 2.0)	0.2	0.9 (-0.3, 2.0)	0.2	0.5 (-0.7, 1.6)	0.4	0.6
Model 2†	1.1 (-0.05, 2.3)	0.06	0.5 (-0.7, 1.7)	0.5	0.9 (-0.3, 2.1)	0.1	0.6 (-0.6, 1.9)	0.3	0.4
Snack									
Model 1*	0.2 (-0.9, 1.4)	0.7	0.3 (-0.9, 1.5)	0.6	0.4 (-0.6, 1.7)	0.4	1.2 (0.02, 2.4)	***0.05***	***0.05***
Males	-0.2 (-1.8, 1.5)	0.8	-0.3 (-1.8, 1.1)	0.7	-0.5 (-2.1, 1.1)	0.5	-0.4 (-2.0, 1.2)	0.6	0.5
Females	0.4 (-1.2, 2.0)	0.6	0.9 (-0.9, 2.6)	0.4	1.0 (-0.6, 2.6)	0.2	2.0 (0.4, 3.6)	***0.01***	***0.01***
Model 2†	0.4 (-0.8, 1.6)	0.5	0.5 (-0.7, 1.7)	0.5	0.1 (-1.1, 1.3)	0.8	1.0 (-0.3, 2.2)	0.1	0.2
Males	-0.2 (-0.2, 1.6))	0.9	-0.3 (-1.9, 1.4)	0.8	-0.8 (-2.7, 0.9)	0.3	-0.5 (-2.4, 1.3)	0.6	0.4
Females	0.5 (-1.1, 2.1)	0.5	0.5 (-1.3, 2.3)	0.6	0.4 -1.1, 2.0)	0.6	1.6 (0, 3.3)	0.05	0.1

*Unadjusted,† Adjusted for age, sex, WHR, physical activity, social class, smoking practice,mean arterial blood pressure, LDL cholesterol, triglycerides and energy intake. All measurements based on fasting concentration.

Table 8-12 Pearson's correlation coefficients for relationship between arterial IMT and distensibility with CVD risk factors (blood pressure, heart rate and obesity)

	SBP (mm Hg)	*DBP (mm Hg)*	*MAP (mm Hg)*	*Heart Rate (BPM)*	*Waist Circumference*	*Waist to Hip Ratio*	*BMI*
Common Carotid Artery							
IMT	0.31**	0.26**	0.31**	-0.02	-0.23**	-0.13*	0.05
Distensibility	-0.45**	-0.30**	-0.39**	-0.20**	-0.30**	-0.30**	-0.26**
Brachial Artery							
Distensibility	-0.28**	-0.21**	-0.25**	-0.10	-0.18**	-0.24**	-0.13

Table 8-13 Pearson's correlation coefficients for relationship between arterial IMT and distensibility with CVD risk factors (serum lipids, glucose and insulin)

	Glucose (mmol/L)	*Insulin (pmol/L)*	*Total Cholesterol (mmol/L)*	*HDL Cholesterol (mmol/L)*	*LDL Cholesterol (mmol/L)*	*VLDL Cholesterol (mmol/L)*	*TAG (mmol/L)*
Common Carotid Artery							
IMT	0.15**	0.06	0.08	0.13*	-0.12*	0.08	0.08
Distensibility	-0.28**	-0.20**	-0.17**	0.10*	-0.21**	-0.16**	-0.20**
Brachial Artery							
Distensibility	-0.21**	-0.16*	-0.10	0.04	-0.14*	-0.20**	-0.20**

** correlation significant at the 0.01 and * 0.05 level (2-tailed).

Abbreviations in Tables: CCA-IMT, common carotid artery intima media thickness, SBP, systolic blood pressure, DBP, diastolic blood pressure, MAP, mean arterial pressure, WC, waist circumference, WHR, waist to hip ratio, BMI, body mass index, Hg, mercury, BPM, beats per minute.

Table 8-14 Univariate linear regression models summarising associations between dietary patterns and Framingham risk factors with measures of vascular health

	Mean CCA IMT				*Brachial Artery Distension (mm)*				*Flow Mediated Dilatation (%)*			
Risk Factor	**β**	**95% CI**	***P***	***R^2***	**β**	**95% CI**	***P***	***R^2***	**β**	**95% CI**	***P***	***R^2***
Age, y	0.006	0.004,0.007	***<0.001***	0.1	-0.001	-0.002, 0.000	*0.1*	0.01	-0.03	0.117, 0.056	*0.5*	-0.002
Men/women	-0.03	-0.04, -0.008	*0.8*	0.03	-0.01	-0.02, 0.002	***0.03***	0.02	2.3	1.4, 3.1	***<0.001***	0.09
SBP, mm Hg	0.002	0.001, 0.003	***<0.001***	0.09	0.000	0.000, 0.001	*0.6*	0.002	-0.02	-0.057, 0.019	*0.3*	0.003
TC, mmol/L	0.007	-0.003, 0.017	*0.6*	0.003	-0.003	-0.008, 0.003	*0.4*	-0.001	-0.09	-0.57, 0.39	*0.7*	-0.003
HDL-C, mmol/L	-0.03	-0.05, -0.004	*0.2*	0.02	-0.02	-0.03, 0.001	*0.06*	0.01	0.08	-0.32, 2.06	*0.2*	0.003
Cotinine, ng/umol	0.000	0.000, 0.000	*0.8*	0.002	0.000	0.000, 0.000	*0.5*	-0.003	-0.004	-0.01, 0.002	*0.2*	0.007
Dietary Pattern												
Healthy	-0.1	-0.012, 0.000	***0.04***	0.01	0.02	-0.004, 0.003	*0.7*	-0.004	0.09	-0.04, 0.55	*0.09*	0.006
Meaty	0.002	-0.004, 0.008	*0.5*	0.002	0.001	-0.004, 0.004	*1.0*	0.000	0.06	-0.45, 0.14	*0.3*	0.003
Snack	0.07	-0.003, 0.01	*0.2*	0.001	-0.1	0.000, 0.007	***0.05***	0.01	-0.01	-0.26, 0.32	*0.8*	0.000

Abbreviations in table: **β**, standardized coefficient, SBP, systolic blood pressure; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol. The R^2 values give the strength of the association between individual risk factors and vascular measures. The coefficient is the strength of the association of the risk factor; for example, a healthy dietary pattern is associated with CCA-IMT such that for every increase in dietary pattern score quintile CCA-IMT decreases by 0.01 mm. For every increase in 1 year of age CCA-IMT increases by 0.005 mm.

Chapter 9

Relationships of *n-3* Fatty Acids with Vascular Structure and Function and Classical CVD Risk Factors

9.1 Introduction

It is well established that diet affects CVD but which nutrients are the most important and there specific roles is unclear. Individual components within dietary patterns have been associated with protective effects for vascular health and CVD risk factors **(Chapter 2, Section 2.3)**. These include *n-3* PUFAs and in particular the *n-3* long-chain fatty acids DHA and EPA. This chapter reports associations of DHA and EPA status, assessed according to erythrocyte membrane concentrations, with measures of vascular structure and function and CVD risk factors.

9.1.1 Sociodemographic Characteristics

Subjects were divided into quintiles of red cell fatty acid concentration, expressed as a percentage total red cell membrane fatty acids (%RBCFA). Subjects were closely matched for demographic variables according to quintiles of fatty acid status **(Tables 9-1 and 9-2)**. Individuals in the highest quintiles for DHA and EPA %RBCFA were suggested to have healthier lifestyles demonstrated by a lower prevalence of smoking in the highest compared with lowest quintile (current smokers in lowest and highest quintiles of DHA status 25 (40%) and 6 (10%) respectively, $P = 0.001$).

9.1.2 Fatty Acid Status and Vascular Structure and Function

Vascular structure and function were related to fatty acid status with different effects according to gender.

9.1.2.1 FMD and Fatty Acid Status

FMD decreased as DHA %RBCFA increased ($P = 0.05$) **(Table 9-3)**. This effect was confined to females (DHA %RBCFA x gender interaction on FMD: $P = 0.003$). In

secondary analyses absolute FMD of the brachial artery adjusted for baseline diameter, skin and room temperature was 0.08 mm (27%) lower for women in the highest quintile of DHA %RBCFA compared with those in the lowest (mean difference between highest and lowest quintile -0.08, 95% CI: -0.1 - 0.08 [*P* = 0.001]) **(Table 9-5, Model 1)**. This effect remained significant in a model fully adjusted for factors known to influence vascular health (age, social class, physical activity, LDL cholesterol, TG, WHR, fasting insulin and energy intake (*P* for trend = 0.008) **(Table 9-5, Model 2).**

9.1.2.2 Arterial distension and fatty acid status

Brachial and CCA distension increased as EPA status increased (*P* for trend = 0.02 and 0.03 respectively) **(Table 9-4)**. For brachial artery distension this effect was confined to males (gender x fatty acid quintile interaction on brachial artery distension: *P* = 0.02). In secondary analyses these effects were attenuated and no longer significant after adjustment for confounders **(Table 9-5)**.

9.1.3 Fatty Acid Status and Cardiovascular Risk Factors

CVD risk factors including obesity, blood pressure, serum cholesterol, triglycerides and insulin resistance were associated with fatty acid status. Relationships varied according to fatty acid and were independent of gender.

9.1.3.1 Obesity

BMI decreased as DHA %RBCFA increased (*P* for trend = 0.01) **(Table 9-1)**. Central obesity, assessed on waist circumference and WHR, was significantly related to DHA status. Waist circumference and WHR decreased with increasing DHA status (*P* for trend = 0.003 and 0.002) **(Table 9-1)**. These associations were independent of gender (RBC DHA quintile x gender interaction on all variables: [*P* > 0.07]). In secondary analyses that adjusted for factors known to influence obesity risk (age, sex, social class, physical activity, and energy intake) the relationships of DHA with BMI and waist circumference were attenuated. The relationship between DHA and WHR remained significant (*P* = 0.03) **(Table 9-6,**

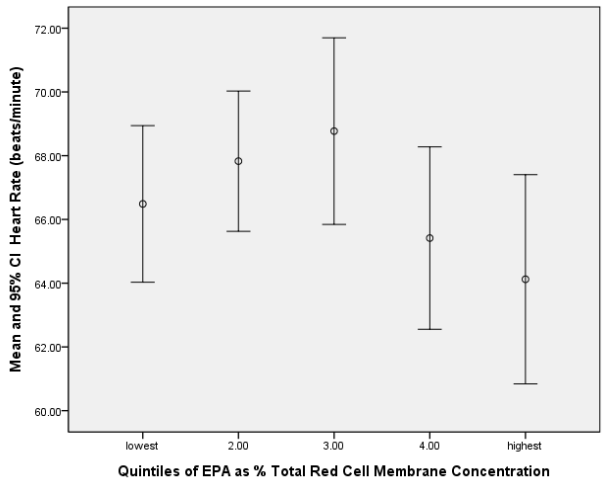
Model 2). No linear trend was found for a relationship of EPA with waist circumference or WHR **(Table 9-6)**.

9.1.3.2 Blood Pressure and Heart Rate

Diastolic blood pressure decreased as DHA and EPA status increased **(Tables 9-1 and 9-2)**. DBP was 5% lower in the highest compared with lowest quintiles for both fatty acids (mean difference: 3 mm Hg, *P* for trend = 0.03 and 0.02 for DHA and EPA respectively). There was no significant interaction of gender with %RBCFA on DBP (*P* > 0.3 for both fatty acids). In secondary analyses relationships were attenuated after adjustment for confounders that potentially influence BP (e.g. obesity and fasting lipids) and no longer significant **(Table 9-7, Model 2)**.

Resting heart rate decreased across quintiles of EPA %RBCFA **(Table 9-2)**. There was no significant interaction of gender with %RBCFA on heart rate (*P* > 0.6) **(Table 9-7, Figure 9-1)**. The effect size was large – heart rate was 3 beats per minute slower in the highest compared with the lowest quintile for EPA %RBCFA. Furthermore, after adjustment for factors known to influence heart rate including gender, physical activity level and CVD risk factors the effect was maintained and showed a significant trend (*P* for trend = 0.05) **(Table 9.7, Model 2)**.

Figure 9-1 Resting heart rate according to quintiles of EPA status



9.1.3.3 Fasting Lipid Concentrations

Total cholesterol, LDL, VLDL and triglyceride concentrations decreased as DHA status increased (*P* for trend < 0.05 for all variables) **(Table 9-1)**. Improvements in total to HDL and LDL to HDL cholesterol ratios were seen with increasing DHA status (*P* for trend = 0.008 and 0.005 respectively) **(Table 9-1)**. There were no significant effects of gender (*P* for gender x %RBCFA quintile interaction: > 0.4 for all variables). In secondary analyses, after adjustment for factors known to influence cholesterol concentrations (e.g. obesity, blood pressure and insulin resistance) effects were attenuated and no longer significant **(Table 9-8)**.

VLDL cholesterol and triglycerides were lower in association with higher EPA status (*P* for trend=0.004 for both variables) **(Table 9-2)**. There were no significant interactions between gender and EPA status for either outcome (*P* = 0.06). The effect size was large (mean difference between highest and lowest quintiles: -0.1mmol/L (25%) and -0.3mmol/L (30%) for VLDL and TAG respectively (*P* for difference = 0.004 for both outcomes) **(Table 9-2)**. Effects were attenuated after adjustment for potential confounders but a trend remained (*P* for trend = 0.06 for both outcomes) **(Table 9-8, Model 2)**.

9.1.3.4 Fasting Glucose and Insulin

Fasting glucose concentration was lower in association with DHA status (difference between highest and lowest DHA quintiles 0.3mmol/L [*P* for trend < 0.001]) **(Table 9-1)**. There was no significant effect of gender (RBC DHA quintile x gender interaction on glucose: [*P* = 0.9]). The strength of the association was reduced after adjustment for confounding factors but remained significant (*P* for trend = 0.001) **(Table 9-9, Model 2)**.

There was no association of fasting glucose with EPA status. However fasting insulin concentration decreased as EPA status increased (*P* for trend = 0.008) **(Table 9-2)**. The difference in fasting insulin concentration between the highest and lowest quintiles of EPA %RBCFA was 5pmol/L (20%) **(Table 9-2)**. Insulin resistance, as measured by HOMA also decreased with increasing EPA status (*P* for trend = 0.02) **(Table 9-2)**. After adjustment for potential confounders relationships of EPA status to blood glucose and insulin resistance were no longer significant **(Table 9-9, Model 2)**.

9.2 *n-3* Fatty Acid Intake and Cardiovascular Health

Vascular health was not directly associated with dietary fatty acid intake assessed by EPIC or the new FishFQ. However, *n-3* fatty acid intake, estimated with the Fish FFQ, was associated with fasting serum VLDL cholesterol and triglyceride concentrations. VLDL and triglycerides were lower in higher quintiles of *n-3* intake, according to this FFQ, for both DHA and EPA (VLDL: *P* for trend between lowest and highest quintiles: 0.03 and 0.005 and TAG 0.03 and 0.004 for DHA and EPA respectively) (data not presented).

9.3 Summary of Results

This observational dietary study found significant relationships between *n-3* fatty acid status and vascular function. FMD was lower for women with higher DHA status. This suggests DHA has gender effects and that these may further be dose

responsive. Therefore, data from this study do not support the hypothesis that *n-3* fatty acids have direct protective effects on FMD in healthy young people.

We also found a significant reduction in heart rate in association with EPA. This effect was maintained after full adjustment for factors known to influence heart rate, including gender, height, and smoking practice. The effect size was similar to that reported previously but in the present study is seen in a younger population.[274]

The present study also found benefits for the lipid profile in association with higher DHA and EPA status. TC, VLDL and TG decreased as DHA status increased and VLDL and triglycerides both decreased significantly across quintiles of EPA status. Most effects were attenuated and no longer significant after adjustment for potential confounding factors. However, a trend remained for the relationship of EPA with VLDL and TG. Both lipids decreased in association with higher EPA status. This suggests that EPA may be one component within a healthy dietary pattern that reduces CVD risk.

Fasting blood glucose concentration was lower in association with higher DHA status suggesting possible benefits of DHA for risk markers of T2DM.

The study found important gender differences in relationships between *n-3* fatty acids and vascular health which warrant further investigation. Mechanisms are unclear and are discussed in the concluding chapter where the implications of these findings for clinical practice are discussed and recommendations for future research are made.

Table 9-1 Subject characteristics at baseline according to quintiles of DHA status

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Erythrocyte DHA, %	**63**	3.1 (0.7)	**62**	4.5 (0.3)	**66**	5.3 (0.2)	**61**	6.2 (0.3)	**62**	7.8 (1.0)	
Males	**29**	3.2 (0.7)	**25**	4.4 (0.3)	**23**	5.3 (0.2)	**20**	6.2 (0.3)	**22**	8 (1.3)	
Females	**34**	3.2 (0.7)	**37**	4.5 (0.3)	**43**	5.3 (0.2)	**40**	6.2 (0.3)	**41**	7.8 (0.8)	
[1]**Male gender, %, (n)**		45 (29)		40 (25)		35 (23)		33 (20)		35 (22)	0.07
Age, y		28.6 (4.5)		28.2 (4.3)		27.7 (5.1)		27.3 (4.8)		27.5 (4.8)	0.1
Males		28.8 (4.9		28.6 (4.4)		28.8 (5.1)		29.4 (4.7)		29.5 (4.8)	0.5
Females		28.5 (4.2)		28 (4.3)		27 (5)		26.2 (4.6)		26.4 (4.5)	***0.01***
[1]**Education:**											
with degree, %, (n)		64 (41)		69 (43)		68 (45)		67 (41)		62 (39)	1
Males	**29**	59 (17)	**25**	68 (17)	**23**	48 (11)	**20**	70 (14)	**22**	73 (16)	0.5
Females	**35**	69 (24)	**37**	70 (26)	**43**	79 (34)	**40**	68 (27)	**40**	58 (23)	0.5
[1]**Social class:**											
Non-manual, %, (n)		64 (41)		69 (43)		53 (35)		46 (28)		51 (32)	***0.01***
Males		55 (16)		44 (16)		44 (10)		60 (12)		59 (13)	0.9
Females		71 (25)		73 (27)		58 (25)		39 (16)		59 (19)	***0.001***
[1]**Current Smoker, %, (n)**		25 (16)		18 (11)		15 (10)		8 (5)		6 (4)	***0.001***
Males		38 (11)		24 (6)		30 (7)		40 (8)		36 (8)	0.7
Females		47 (16)		43 (16)		60 (26)		32 (13)		32 (13)	***0.04***
[2]**Cotinine, ng/ml**	**44**	3.3 (284)	**48**	2.0 (263)	**38**	1.3 (222)	**30**	1.1 (222)	**34**	1 (238)	***0.02***
Males		4.1 (322)		1 (217)		0.6 (1.8)		2.3 (3.2)		2.2 (3.2)	0.3
Females		2.8 (260)		3.4 (284)		2.3 (273)		0.8 (165)		0.5 (246)	***0.04***
Physical activity level, hours/day	**64**	2 (2.3)	**62**	1.2 (0.8)	**65**	1.8 (2.1)	**61**	1.7 (1.8)	**63**	1.7 (2.0)	0.9
Males		2.6 (2.4)		1.6 (1.6)		2.2 (2.3)		2.1 (1.9)		2.1 (1.9)	0.7

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Females		1.5 (2)		1 (1.8)		1.6 (2)		1.5 (1.7)		1.4 (1.8)	0.6
Anthropometry and body composition	**64**		**62**		**66**		**62**		**64**		
Males	**29**		**25**		**23**		**25**		**29**		
Females	**35**		**37**		**43**		**37**		**35**		
Body Mass Index, kg/m^2		24.5 (4.5)		24 (4)		23.6 (4)		22.7 (3.0)		23.1 (3.6)	***0.01***
Males		24.2 (2.7)		24.4 (2.8)		24.5 (.3.)		23.1 (2.1)		24.6 (3.7)	0.8
Females		24.7 (5.6)		23.7 (5)		23.2 (4.3)		22.5 (3.3)		22.4 (3.3)	***0.008***
Waist (cm)		80 (11.9)		78.8 (10.4)		77.3 (9.6)		75 (9.6)		75.8 (9.5)	***0.003***
Males		84.2 (7.4)		84 (7.6)		85 (9)		81 (6.5)		82.3 (9.8)	0.2
Females		76.3 (13.*)		75.2 (10.5)		23.1 (7)		72 (7.8)		72.3 (7.3)	***0.02***
Waist:Hip ratio		0.8 (0.08)		0.79 (0.06)		0.78 (0.08)		0.77 (0.07)		0.76 (0.06)	***0.002***
Males		0.9 (0.05)		0.8 (0.05)		0.8 (0.07)		0.8 (0.05)		0.8 (0.06)	***0.02***
Females		0.8 (0.06)		0.8 (0.05)		0.7 (0.05)		0.7 (0.06)		0.7 (0.03)	0.1
[2]Sum skinfold, mm		49.4 (44)		49.2 (39)		49.8 (39)		42.8 (38)		46.5 (38)	0.09
Males		42.1(42)		46.1 (41)		41.7 (33)		34.5 (39)		38.9 (46)	0.1
Females		56.8 (41)		51.4 (37)		55.1 (35)		48 (40)		50 (38)	0.1
% Fat (sum skinfolds)		24.5 (5.3)		23.4 (5.7)		24.4 (5.3)		24.3 (6.1)		23.3 (5.4)	0.4
Males		23.3 (4.8)		22.6 (6)		24.8 (5.3)		23.10 (5.5)		23.3 (4.8)	0.8
Females		25.6 (5.5)		24 (5.4)		24 (5.3)		24.8 (6.5)		23.3 (5.7)	0.2
Blood pressure, mm Hg											
Systolic	**64**	112 (11)	**62**	113 (10)	**65**	113 (11)	**61**	112 (12)	**62**	110 (10)	0.3
Males	**29**	115 (11)	**25**	116 (10)	**23**	118 (10)	**20**	117 (11)	**22**	116 (7)	0.6
Females	**35**	110 (10)	**37**	11 (10)	**42**	109 (11)	**41**	110 (11)	**40**	106 (10)	0.3
Diastolic		67 (7)		68 (8)		67 (7)		66 (9)		64 (8)	***0.03***

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Males		68 (7)		69 (7)		70 (8)		68 (10)		67 (7)	0.8
Females		66 (7)		67 (8)		66 (7)		65 (8)		62 (8)	***0.03***
Mean arterial pressure		84 (7)		84 (8)		85 (9)		84 (11)		82 (9)	0.3
Males		86 (6)		87 (7)		89 (10)		88 (11)		86 (6)	0.8
Females		82 (7)		82 (8)		83 (8)		82 (10)		79 (9)	0.3
Resting heart rate, beats/min)		67 (9)		68 (10)		68 (11)		67 (11)		65 (14)	0.4
Males		64 (9)		65 (12)		67 (13)		62 (7)		66 (18)	0.4
Females		67 (9)		69 (9)		68 (10)		70 (11)		64 (11)	0.4
†**Total cholesterol, mmol/L**	**64**	4.3 (1)	**61**	4.3 (0.7)	**66**	4.4 (0.9)	**60**	4.1 (0.7)	**62**	4.1 (0.7)	***0.03***
Males	**29**	4.4 (1)	**24**	4.3 (0.5)	**23**	4.3 (1)	**20**	4.3 (0.8)	**21**	4.3 (0.8)	0.3
Females	**35**	4.2 (0.9)	**37**	4.2 (0.8)	**43**	4.4 (0.9)	**40**	4 (0.7)	**41**	4 (0.7)	0.09
†**LDL cholesterol, mmol/L**		2.5 (0.8)		2.4 (0.7)		2.4 (0.9)		2.3 (0.7)		2.2 (0.7)	***0.03***
Males		2.6 (0.8)		2.6 (0.5)		2.6 (0.9)		2.6 (0.8)		2.5 (0.7)	0.6
Females		2.4 (0.8)		2.3 (0.8)		2.4 (0.9)		2.2 (0.6)		2.1 (0.7)	***0.03***
†**HDL cholesterol, mmol/L**		1.4 (0.4)		1.4 (0.4)		1.5 (0.4)		1.4 (0.3)		1.5 (0.3)	0.2
Males		1.3 (0.3)		1.2 (0.3)		1.3 (0.4)		1.3 (0.3)		1.3 (0.3)	0.7
Females		1.5 (0.4)		1.5 (0.4)		1.7 (0.3)		1.4 (0.3)		1.6 (0.3)	0.6
†[2]**VLDL cholesterol**		0.4 (54)		0.2 (46)		0.4 (44)		0.4 (57)		0.4 (47)	***0.04***
Males		0.5 (47)		0.5 (56)		0.3 (49)		0.4 (71)		0.4 (52)	0.5
Females		0.4 (60)		0.4 (39)		0.4 (41)		0.4 (49)		0.3 (43)	***0.03***
Total:HDL cholesterol ratio		3.3 (1.2)		3.3 (1.1)		3.0 (1.0)		3.1 (0.8)		2.9 (0.8)	***0.008***
Males		3.5 (1)		3.8 (1)		3.5 (1.2)		3.5 (0.9)		3.4 (1)	0.4
Females		3.2 (1.3)		3 (1)		2.8 (0.7)		2.9 (0.7)		2.7 (0.6)	***0.02***
LDL:HDL cholesterol ratio		2.0 (0.9)		1.9 (0.9)		1.7 (0.9)		1.6 (0.7)		1.8 (0.8)	***0.005***

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p‡
Males		2.1 (0.8)		2.2 (0.8)		2.2 (1)		2.1 (0.7)		2 (0.8)	0.4
Females		1.9 (1)		1.7 (0.9)		1.5 (0.7)		1.6 (0.6)		1.4 (0.6)	***0.01***
†2**Trigycerides, mmol/L**		0.5 (54)		0.9 (46)		0.8 (43)		0.8 (57)		0.8 (47)	***0.04***
Males		1 (47)		0.8 (56)		0.8 (49)		0.8 (71)		0.8 (52)	0.6
Females		0.9 (0.6)		0.9 (0.4)		0.8 (0.4)		0.7 (0.5)		0.8 (0.4)	***0.03***
†2**Glucose, mmol/L**		4.9 (0.5)		4.9 (0.5)		4.8 (0.5)		4.7 (0.4)		4.6 (0.5)	***<0.001***
Males		5.1 (0.6)		5 (0.4)		5 (0.4)		4.9 (0.4)		4.8 (0.5)	***0.02***
Females		4.8 (0.5)		4.9 (0.5)		4.6 (0.4)		4.6 (0.4)		4.6 (0.5)	***0.001***
†2**Insulin, pmol/L**		30.6 (74)		27.8 (63)		31.3 (47)		31.3 (55)		31.3 (55)	0.2
Males		27 (69)		25.7 (58)		27.8 (53)		29.9 (54)		29.9 (52)	0.8
Females		33.4 (78)		30.6 (66)		31.3 (44)		32 (56)		30 (57)	0.2
†2**Insulin resistance, (HOMA)**		1 (84)		0.9 (68)		0.9 (52)		0.5 (58)		0.9 (56)	0.09
Males		0.9 (77)		0.8 (62)		0.9 (58)		0.9 (61)		0.9 (57)	0.6
Females		1 (84)		0.9 (72)		0.9 (48)		1 (57)		0.9 (56)	0.08
†2**CRP, mg/L**		1.03 (112)		1.0 (130)		0.8 (110)		1.0 (17)		1.1 (140)	0.7
Males		0.8 (91)		0.8 (103)		0.8 (117)		0.7 (130)		0.9 (147)	0.3
Females		1.2 (125)		1 (144)		0.9 (109)		1.2 (107)		1.1 (139)	0.8

* Quintile 1 is the lowest. Fatty acid status assessed by erythrocyte membrane concentration and expressed as % total fatty acids (RBCFA%). ‡ P for trend calculated with RBCFA% quintile modeled on a continuous scale in general linear models. Data are mean, SD, except: 1% (n) and 2geometric mean (coefficient of variation). † Measurements obtained after 12 hours fasting.

Table 9-2 Subject characteristics at baseline according to quintiles of EPA status

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p ‡
Erythrocyte EPA, %	**63**	0.5 (0.1)	**65**	0.7 (0.05)	**67**	0.9 (0.06)	**58**	1.1 (1.0)	**63**	1.8 (0.50)	
Males	**23**	0.5 (0.1)	**25**	0.7 (0.04)	**19**	0.8 (0.07)	**26**	1.1 (0.09)	**26**	1.9 (0.7)	
Females	**40**	0.5 (0.09)	**40**	0.7 (0.05)	**48**	0.9 (0.06)	**32**	1.1 (0.1)	**37**	1.8 (0.4)	
[1]Male gender, %, (n)		37 (23)		38 (25)		28 (19)		45 (26		41 (26)	***0.02***
Age, y		27.7 (4.6)		27.6 (4.6)		28.4 (4.6)		27.9 (4.8)		27.7 (4.6)	0.9
Males		28.3 (4.6)		29.1 (4.7)		29.9 (4.8)		28.7 (4.6)		29 (5)	0.7
Females		27.3 (4.5)		26.7 (4.2)		27.8 (4.4)		27.2 (5)		26.8 (5.2)	0.8
[1]Education:											
with degree, %, (n)		70 (44)		71 (46)		69 (46)		48 (28)		71 (45)	0.6
Males		65 (15)		64 (16)		68 (13)		46 (12)		73 (19)	0.8
Females		73 (29)		75 (30)		72 (33)		50 (16)		70 (26)	0.6
[1]Social class:											
Non-manual, %, (n)		56 (35)		63 (41)		55 (37)		57 (33)		52 (33)	0.5
Males		43 (10)		60 (15)		68 (13)		62 (16)		50 (13)	0.7
Females		62 (25)		65 (26)		50 (24)		53 (17)		54 (20)	0.3
[1]Current Smoker, %, (n)		48 (30)		45 (29)		37 (25)		33 (19)		33 (21)	***0.004***
Males		39 (9)		36 (9)		21 (4)		35 (9)		35 (9)	0.1
Females		54 (21)		50 (20)		44 (20)		31 (10)		32 (12)	***0.02***
[2]Cotinine, ng/ml		3.4 (278)		2.0 (259)		0.8 (234)		2.2 (247)		0.8 (240)	***0.04***
Males		3.5 (325)		1 (197)		0.5 (188)		3.3 (297)		1.2 (290)	1.0
Females		3.4 (255)		3 (286)		1.1 (253)		1.7 (202)		0.6 (178)	***0.04***
Physical activity level, hours/day		1.92 (2.3)		1.4 (1.9)		1.7 (2)		1.5 (1.8)		1.7 (0.1)	0.8

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p ‡
Males		2.4 (2.4)		2.3 (2.4)		2.2 (2.2)		1.9 (1.7)		2 (1.7)	0.3
Females		1.7 (2.3)		0.9 (1.3)		1.5 (1.9)		1.3 (1.8)		1.6 (1.7)	0.7
Anthropometry and body composition											
Body Mass Index, kg/m2		24.1 (4.5)		23.5 (4.3)		24.2 (4.2)		22.8 (3.0)		23.3 (3.3)	0.1
Males		24 (2.5)		24.6 (2.8)		25.2 (2.6)		23.4 (3.3)		24.1 (3.3)	0.6
Females		24.2 (5.3)		22.8 (5)		24 (4.6)		22.3 (2.7)		22.7 (3.3)	0.1
Waist (cm)		79 (11.9)		76 (10.2)		78.9 (10.4)		76.7 (8.2)		76.2 (9.3)	0.2
Males		84.7 (6.6)		84.1 (8.2)		86 (7.9)		81.4 (8.6)		82 (8.7)	0.1
Females		75 7 (13.1)		70.9 (7.8)		75.9 (9.9)		72.8 (5.5)		72.2 (7.6)	0.3
Waist:Hip ratio		0.79 (0.07)		0.78 (0.08)		0.79 (0.07)		0.77 (0.06)		0.77 (0.06)	0.2
Males		0.9 (0.04)		0.9 (0.06)		0.84 (0.06)		0.83 (0.06)		0.81 (0.05)	0.1
Females		0.8 (0.06)		0.7 (0.05)		0.8 (0.05)		0.7 (0.04)		0.7 (0.04)	0.3
^{2}Sum skinfold, mm		48.5 (42)		46.2 (40)		53.4 (37)		42 (44)		47.5 (40)	0.4
Males		40.4 (43)		39.3 (40)		49.4 (33)		36.6 (45)		41 (38)	0.8
Females		54.1 (38)		51.4 (36)		55.1 (39)		50 (39)		52.4 (38)	0.5
% Fat (sum skinfold)		24.2 (4.8)		23.8 (5.6)		23 (4.9)		24.6 (5.6)		24 (5.5)	0.6
Males		23.8 (4.9)		23.4 (5.6)		21.6 (4.1)		23.7 (6.4)		24 (4.8)	0.8
Females		24.4 (4.8)		24 (5.6)		23.5 (5.1)		25.3 (6.8)		24.6 (6.2)	0.6
Blood pressure, mm Hg											
Systolic	**63**	110 (10)	**65**	113 (10)	**67**	113 (13)	**58**	112 (11)	**62**	111 (10)	0.8
Males	**23**	114 (11)	**25**	118 (10)	**19**	119 (10)	**26**	117 (10)	**26**	115 (8)	1.0
Females	**40**	108 (10)	**40**	110 (8)	**48**	111 (13)	**32**	107 (10)	**36**	108 (11)	0.5
Diastolic		67 (6)		67 (7)		68 (9)		66 (8)		64 (8)	***0.02***
Males		69 (6)		68 (8)		72 (8)		68 (8)		66 (7)	0.3

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p ‡
Females		66 (7)		66 (6)		67 (10)		64 (7)		63 (9)	***0.02***
Mean arterial pressure		83 (7)		85 (8)		85 (11)		84 (10)		82 (9)	0.3
Males		87 (6)		87 (10)		90 (7)		88 (9)		85 (7)	0.7
Females		82 (7)		83 (7)		83 (11)		80 (9)		80 (9)	0.2
Resting heart rate, beats/min)		66 (10)		68 (9)		69 (12)		65 (11)		64 (13)	***0.03***
Males		64 (9)		66 (10)		66 (11)		65 (13)		64 (10)	0.3
Females		68 (10)		69 (8)		70 (12)		66 (9)		64 (9)	***0.05***
†**Total cholesterol, mmol/L**	**63**	4.2 (0.9)	**64**	4.4 (0.8)	**65**	4.2 (0.8)	**58**	4.2 (0.8)	**62**	4.2 (0.8)	0.2
Males	**23**	4.3 (0.7)	**24**	4.5 (1.1)	**19**	4.2 (0.7)	**26**	4.3 (0.8)	**25**	4.4 (0.8)	0.5
Females	**40**	4.2 (1)	**40**	4.3 (0.7)	**47**	4.3 (0.9)	**32**	4.1 (0.8)	**37**	4 (0.7)	0.2
†**LDL cholesterol, mmol/L**		2.4 (0.8)		2.4 (0.7)		2.4 (0.7)		2.2 (0.9)		2.3 (0.7)	0.4
Males		2.5 (0.7)		2.6 (0.9)		2.6 (0.6)		2.5 (0.9)		2.6 (0.7)	0.8
Females		2.3 (0.8)		2.3 (0.5)		2.4 (0.8)		2 (0.8)		2.2 (0.7)	0.1
†**HDL cholesterol, mmol/L**		1.4 (0.4)		1.5 (0.3)		1.4 (0.4)		1.5 (0.3)		1.5 (0.3)	0.3
Males		1.3 (0.2)		1.3 (0.3)		1.1 (0.2)		1.3 (0.3)		1.4 (0.3)	0.2
Females		1.5 (0.4)		1.6 (0.3)		1.5 (0.4)		1.6 (0.3)		1.5 90.3)	0.4
†[2]**VLDL cholesterol**		0.4 (53)		0.4 (49)		0.4 (48)		0.4 (51)		0.3 (47)	***0.004***
Males		0.5 (53)		0.4 (58)		0.4 (47)		0.4 (58)		0.4 (57)	0.2
Females		0.4 (53)		0.4 (42)		0.4 (48)		0.4 (46)		0.3 (38)	***0.003***
Total:HDL cholesterol ratio		3.2 (1.2)		3.1 (0.9)		3.3 (1.0)		3.0 (1.0)		3.0 (1.0)	0.1
Males		3.5 (0.9)		3.6 (1.1)		3.9 (0.8)		3.4 (1.1)		3.3 (1)	0.5
Females		3.1 (1.3)		2.8 (0.5)		3.1 (0.9)		2.6 (0.8)		2.7 (0.7)	***0.05***
LDL:HDL cholesterol ratio		1.8 (0.9)		1.8 (0.7)		1.9 (0.8)		1.6 (0.9)		1.7 (0.7)	0.2
Males		2 (0.7)		2.1 (0.9)		2.4 (07)		2 (0.9)		2 (0.8)	0.7
Females		1.8 (1)		1.5 (0.5)		1.7 (0.8)		1.3 (0.7)		1.5 (0.6)	0.06

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	p ‡
†[2]**Trigycerides, mmol/L**		1.0 (53)		0.9 (49)		0.9 (48)		0.8 (51)		0.7 (47)	***0.004***
Males		1 (54)		1 (58)		1 (47)		0.8 (58)		0.8 (57)	0.2
Females		1 (53)		0.8 (42)		0.9 (48)		0.8 (46)		0.7 (38)	***0.003***
†[2]**Glucose, mmol/L**		4.8 (0.5)		4.8 (0.5)		4.8 (0.5)		4.9 (0.5)		4.7 (0.5)	0.4
Males		4.9 (0.4)		5 (0.6)		5.1 (0.4)		5 (0.5)		4.9 (0.4)	0.6
Females											0.4
†[2]**Insulin, pmol/L**		32 (74)		29.9(59)		33.4 (63)		33.4 (48)		27 (46)	***0.008***
Males		25.7 (67)		27.8 (71)		30.6 (57)		27 (55)		30 (36)	0.6
Females		35.4 (75)		32 (51)		34.8 (42)		28.5 (51)		25 (60)	***0.007***
†[2]**Insulin resistance, (HOMA)**		1 (78)		1 (64)		1 (67)		0.9 (55)		0.8 (46)	***0.02***
Males		0.8 (71)		0.9 (79)		1 (61)		0.9 (63)		0.9 (39)	0.6
Females		1.1 (81)		0.9 (54)		1 (70)		0.9 (48)		0.7 (49)	***0.01***
†[2]**CRP, mg/L**		6.4 (166)		3.1 (150)		7.6 (154)		6.7 (203)		5.9 (192)	0.7
Males		0.8 (100)		0.7 (95)		0.6 (110)		0.8 (133)		1.1 (132)	***0.06***
Females		1.2 (127)		1.1 (105)		1.2 (119)		1.2 (136)		0.8 (139)	0.7

* Quintile 1 is the lowest. Fatty acid status assessed by erythrocyte membrane concentration and expressed as % total fatty acids (RBCFA%). ‡ *P* for trend calculated with RBCFA% quintile modelled on a continuous scale in general linear models. Data are mean, SD, except: [1]% (n) and [2]geometric mean (coefficient of variation). † Measurements obtained after 12 hours fasting.

Table 9-3 Vascular variables at baseline according to quintiles of DHA status

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	P ‡
Right Brachial Artery											
Diameter, mm	**64**	3.4 (0.5)	**62**	3.2 (0.5)	**65**	3.2 (0.6)	**60**	3.1 (0.6)	**60**	3.2 (0.5)	***0.03***
Males	**29**	3.8 (0.4)	**25**	3.6 (0.4)	**23**	3.8 (0.4)	**20**	3.7 (0.5)	**21**	3.7 (0.4)	0.7
Females	**35**	3 (0.3)	**37**	2.9 (0.3)	**42**	2.9 (0.4)	**40**	2.8 (0.4)	**39**	2.9 (0.2)	0.07
Reactive hyperaemia, %		746 (255)		701 (288)		720 (250)		758 (237		754 (293)	0.5
Males		625 (170)		606 (304)		620 (178)		701 (287)		647 (170)	0.4
Females		852 (272)		762 (265)		771 (268)		788 (207)		814 (331)	0.8
Flow-mediated dilatation, mm		0.28 (0.1)		0.27 (0.1)		0.28 (0.1)		0.28 (0.1)		0.25 (0.1)	***0.05***
Males		0.26 (0.1)		0.26 (0.07)		0.28 (0.1)		0.29 (0.1)		0.28 (0.1)	0.2
Females		0.3 (0.1)		0.27 (0.09)		0.28 (0.1)		0.28 (0.1)		0.23 (0.09)	***0.003***
Flow-mediated dilatation, %		8.4 (3.8)		8.2 (3.3)		8.5 (3.9)		9.0 (4.1)		7.6 (3.6)	0.4
Males		6.5 (2.9)		6.8 (2.4)		6.9 (3.6)		7.7 (3.2)		7.3 (4.2)	0.2
Females		10.1 (3.6)		9.1 (3.5)		9.4 (3.9)		9.7 (4.3)		7.8 (3.2)	***0.04***
Distension, mm	**41**	0.09 (0.03)	**44**	0.09 (0.04)	**44**	0.09 (0.04)	**43**	0.08 (0.03)	**41**	0.1 (0.04)	0.5
Males	**20**	0.09 (0.03)	**14**	0.1 (0.05)	**14**	0.09 (0.04)	**16**	0.08 (0.04)	**15**	0.1 (0.04)	0.8
Females	**21**	0.08 (0.04)	**30**	0.08 (0.04)	**30**	0.08 (0.04)	**27**	0.08 (0.02)	**26**	0.089 (0.05)	0.6
Distension, %		11.4 (4.5)		12.9 (5.6)		12.3 (6.9)		11.4 (4.8)		13.5 (5.8)	0.3
Males		9.7 (3)		12.5 (4.6)		10 (4.1)		8.5 (3.9)		12 (3.8)	0.8
Females		13.1 (5.2)		13.1 (6.1)		13.4 (7.7)		13.1 (4.5)		14.3 (6.6)	0.5
Distension coefficient ($x10^{-3}.kPa^{-1}$)		8.9 (5.2)		9.4 (3.9)		9.0 (4.9)		8.4 (3.5)		10 (4.2)	0.5
Males		8.2 (6.5)		9.2 (3.2)		7.2 (2.9)		6.2 (2.8)		8.9 (2.8)	0.4
Females		9.6 (3.7)		9.5 (4.2)		9.8 (5.4)		9.6 (3.2)		10.6 (4.8)	0.4
Right Common Carotid Artery											

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	P ‡
Diameter, mm	**53**	6.5 (0.5)	**57**	6.5 (0.6)	**60**	6.5 (0.5)	**60**	6.4 (0.5)	**57**	6.4 (0.5)	0.07
Males	**24**	6.8 (0.4)	**21**	6.9 (0.6)	**22**	6.9 (0.6)	**20**	6.8 (0.4)	**21**	6.7 (0.5)	0.6
Females	**29**	6.4 (0.4)	**35**	6.3 (0.4)	**38**	6.3 (0.4)	**40**	6.3 (0.4)	**36**	6.2 (0.4)	0.1
Distension, mm		0.7 (0.1)		0.7 (0.1)		0.7 (0.2)		0.7 (0.2)		0.8 (0.2)	0.4
Males		0.7 (0.2)		0.7 (0.2)		0.8 (0.2)		0.7 (0.2)		0.8 (0.2)	0.6
Females		0.7 (0.1)		0.7 (0.1)		0.7 (0.2)		0.7 (0.2)		0.8 (0.2)	0.3
Distension, %		11.2 (2.5)		11.3 (2.1)		11 (3)		11.6 (2.6)		11.8 (2.6)	0.4
Males		11 (3.3)		10.7 (2.2)		11.2 (3.4)		10.9 (2.8)		11.4 (2.7)	0.9
Females		11.4 (1.7)		11.6 (2)		10.9 (2.8)		11.9 (2.5)		12.1 (2.6)	0.7
Distension coefficient ($x10^{-3}.kPa^{-1}$)		34.8 (13.7)		34.6 (6.7)		33 (8.4)		34 (8)		35 (7.1)	0.9
Males		33.3 (19.2)		32 (6.4)		30.7 (7.5)		30.5 (8.1)		31.2 (5.7)	0.4
Females		36.1 (6.5)		36.2 (6.5)		34.3 (8.6)		35.6 (7.5)		37 (7)	0.7
Intima-media thickness, mm		0.55 (0.09)		0.54 (0.09)		0.56 (0.08)		0.53 (0.06)		0.55 (0.08)	0.8
Males		0.57 (0.09)		0.55 (0.08)		0.58 (0.1)		0.54 (0.06)		0.57 (0.1)	0.9
Females		0.53 (0.07)		0.54 (0.09)		0.5 (0.05)		0.53 (0.05)		0.5 (0.07)	0.9
Carotid-radial PWV m/s		8.7 (1.1)		8.5 (1)		8.8 (1.1)		8.7 (1.8)		8.3 (1.2)	0.2
Males		8.6 (1.1)		8.6 (1)		9.1 (1)		9.2 (1.4)		8.6 (1.3)	0.5
Females		8.8 (1.1)		8.4 (1)		8.6 (1.2)		8.5 (1.9)		8.1 (1.1)	0.06

* Quintile 1 is the lowest. Fatty acid status assessed by erythrocyte membrane concentration and expressed as % total fatty acids (RBCFA%). ‡ *P* for trend calculated with RBCFA% quintile modelled on a continuous scale in general linear models. Data are mean, SD.

Table 9-4 Vascular variables at baseline according to quintiles of EPA status

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	P ‡
Right Brachial Artery											
Diameter, mm	**62**	3.2 (0.5)	**65**	3.2 (0.6)	**66**	3.2 (0.6)	**58**	3.3 (0.6)	**60**	3.2 (0.5)	0.9
Males	**23**	3.7 (0.5)	**25**	3.8 (0.4)	**19**	3.8 (0.4)	**26**	3.7 (0.4)	**25**	3.7 (0.4)	0.4
Females	**39**	2.9 (0.3)	**40**	2.9 (0.4)	**47**	2.9 (0.3)	**32**	2.9 (0.4)	**35**	2.9 (0.3)	0.9
Reactive hyperaemia, %		790 (269)		736 (263)		707 (241)		737 (227)		716 (316)	0.2
Males		630 (211)		636 (263)		656 (210)		672 (256)		602 (188)	0.8
Females		879 (259)		800 (246)		728 (252)		788 (190)		800 (363)	0.2
Flow-mediated dilatation, mm		0.27 (0.1)		0.27 (0.1)		0.3 (0.1)		0.27 (0.1)		0.25 (0.1)	0.2
Males		0.2 (0.09)		0.3 (0.1)		0.3 (0.09)		0.3 (0.1)		0.3 (0.1)	0.6
Females		0.29 (0.1)		0.26 (0.1)		0.3 (0.1)		0.3 (0.1)		0.2 (0.08)	***0.03***
Flow-mediated dilatation, %		8.2 (3.6)		8.3 (3.7)		9.5 (4.1)		8 (3.5)		7.8 (3.6)	0.3
Males		6 (2.8)		7.2 (2.9)		6.9 (2.6)		7.1 (3.2)		7.6 (4.3)	0.5
Females		9.5 (3.4)		8.9 (3.9)		10.5 (4.2)		8.7 (3.6)		7.9 (3)	0.1
Right Brachial Artery Distension											
Distension, mm	**37**	0.08 (0.03)	**52**	0.09 (0.04)	**49**	0.08 (0.04)	**35**	0.08 (0.03)	**40**	0.1 (0.04)	***0.02***
Males	**13**	0.09 (0.04)	**20**	0.09 (0.04)	**13**	0.08 (0.05)	**17**	0.09 (0.03)	**16**	0.1 (0.03)	***0.05***
Females	**24**	0.07 (0.03)	**32**	0.09 (0.05)	**36**	0.08 (0.03)	**18**	0.07 (0.02)	**24**	0.1 (0.04)	0.2
Distension, %		11.3 (4.3)		13 (7.5)		11.3 (4.6)		11 (4.1)		14.6 (5.5)	0.08
Males		10 1 (3.8)		9.7 (4.1)		9 (4.5)		10.4 (3.9)		12.9 (3)	***0.02***
Females		11.9 (4.5)		15.2 (8.4)		12.2 (4.4)		11.6 (4.3)		15.7 (6.5)	0.3
Distension coefficient ($x10^{-3}.kPa^{-1}$)		8.9 (5.3)		9.5 (5.2)		8.3 (3.3)		8.1 (3)		10.7 (4)	0.2
Males		9.1 (8)		7.1 (3)		6.5 (3.2)		7.6 (2.8)		9.5 (2.2)	0.4
Females		8.8 (3.3)		11 95.7)		9 (8.1)		8.5 (3.1)		11.5 (4.7)	0.3

	n	*Q1**	*n*	*Q2*	*n*	*Q3*	*n*	*Q4*	*n*	*Q5*	P ‡
Right Common Carotid Artery											
Diameter, mm	**55**	6.5 (0.5)	**59**	6.4 (0.5)	**61**	6.5 (0.5)	**53**	6.5 (0.5)	**59**	6.5 (0.5)	0.7
Males	**20**	6.7 (0.4)	**22**	6.8 (0.4)	**17**	7 (0.5)	**26**	6.8 (0.5)	**24**	6.8 (0.5)	0.5
Females	**35**	6.4 (0.5)	**37**	6.1 (0.4)	**44**	6.3 (0.4)	**27**	6.3 (0.4)	**35**	6.3 (0.4)	0.6
Distension, mm		0.7 (0.2)		0.7 (0.1)		0.7 (0.1)		0.7 (0.1)		0.8 (0.2)	***0.03***
Males		0.8 (0.2)		0.7 (0.2)		0.7 (0.2)		0.8 (0.2)		0.8 (0.2)	0.08
Females		0.7 (0.2)		0.7 (0.1)		0.7 (0.1)		0.7 (0.1)		0.8 (0.2)	0.09
Distension, %		11.2 (3)		11.2 (2.2)		11.2 (2.3)		11.2 (2.2)		12.1 (2.9)	0.7
Males		11.4 (3.4)		10.3 (2.5)		9.4 (2.7)		11.3 (2.4)		12.2 (3)	0.3
Females		11 (2.8)		11.6 (1.9)		11.8 (2.1)		11.2 (2)		12 (2.9)	0.9
Distension coefficient ($x10^{-3}.kPa^{-1}$)		36.1 (14)		33.1 (7.3)		33.9 (8.2)		32.9 (6.1)		35.1 (7.2)	0.7
Males		36.4 (20.4)		28.1 (5.8)		27.8 (7.2)		31.4 (6.8)		33.6 (6.2)	0.2
Females		35.9 (9)		36.1 (6.4)		36.1 (7.5)		34.4 (5.1)		36.2 (7.8)	0.9
Intima-media thickness, mm		0.54 (0.06)		0.56 (0.09)		0.54 (0.07)		0.55 (0.09)		0.54 (0.07)	1.0
Males		0.57 (0.05)		0.57 (0.1)		0.54 (0.08)		0.57 (0.1)		0.55 (0.08)	0.7
Females		0.52 (0.06)		0.55 (0.08)		0.54 (0.06)		0.54 (0.07)		0.53 (0.07)	0.9
Carotid-radial PWV m/s		8.7 (1.2)		8.7 (1.3)		8.6 (1.6)		8.4 (1.0)		8.5 (1.2)	0.3
Males		8.7 (1.3)		9.1 (1.4)		8.6 (0.9)		8.5 (0.9)		8.9 (1.3)	0.9
Females		8.7 (1.2)		8.4 (1.2)		8.6 (1.8)		8.3 (1.1)		8.2 (1.1)	0.1

* Quintile 1 is the lowest. Fatty acid status assessed by erythrocyte membrane concentration and expressed as % total fatty acids (RBCFA%). ‡ *P* for trend calculated with RBCFA% quintile modelled on a continuous scale in general linear models. Data are mean, SD.

Table 9-5 Regression coefficient (95% CI) for vascular variables according to quintiles[1] of fatty acids status

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Right brachial artery									
Flow Mediated Dilatation (mm)									
DHA									
Model 1[*] **n=301**	0.02 (-0.05, 0.02)	0.4	0 (-0.03, 0.04)	1	0 (-0.04, 0.04)	1	-0.04 (-0.07, -0.001)	0.04	0.2
Males: **n=115**	-0.01 (-0.07, 0.05)	0.7	0.02 (-0.04, 0.07)	0.5	0.03 (-0.03, 0.09)	0.3	0.02 (-0.04, 0.07)	0.7	0.7
Females: **n= 186**	-0.03 (-0.08, 0.01)	0.2	-0.02 (-0.06, 0.03)	0.4	-0.02 (-0.07, -0.02)	0.3	-0.08 (-0.1, 0.03)	***0.001***	***0.006***
Model 2[†] **n=293**	-0.02 (-0.05, 0.02)	0.4	0.01 (-0.03, 0.05)	0.6	0.004 (-0.03, 0.04)	0.8	-0.03 (-0.06, 0.01)	0.1	0.4
Males: **n= 110**	-0.002 (-0.06, 0.06)	0.9	0.05 (-0.01, 0.1)	0.1	0.04 (-0.02, 0.09)	0.2	0.04 (-0.02 (0.1)	0.2	0.08
Females: **n=183**	-0.04 (-0.08, 0.01)	0.1	-0.01 (-0.06, 0.03)	0.5	-0.03 (-0.07, 0.02)	0.3	-0.07 (-0.1, -0.03)	***0.002***	***0.008***
EPA									
Model 1[*]	0.004 (-0.03, 0.04)	0.8	0.03 (-0.01, 0.06)	0.1	-0.001 (-0.04, 0.04)	1	-0.02 (-0.05, 0.02)	0.3	0.3
Males	0.05 (-0.004, 0.1)	0.07	0.04 (-0.02, 0.1)	0.2	0.03 (-0.03, 0.09)	0.3	0.04 (-0.02, 0.09)	0.2	0.6
Females	-0.03 (-0.07, 0.02)	0.2	0.02 (-0.02, 0.06)	0.4	-0.02 (-0.07, 0.03)	0.4	-0.06 (-0.1, -0.01)	0.02	0.0
Model 2[†]	0.01 (-0.03, 0.04)	0.6	0.03 (-0.01, 0.06)	0.2	0.003 (-0.03, 0.04)	0.9	-0.01 (-0.05, 0.03)	0.6	0.6
Males	0.05 (-0.01, 0.1)	0.1	0.04 (-0.02, 0.1)	0.2	0.04 (-0.03, 0.09)	0.3	0.05 (-0.01, 0.1)	0.1	***0.02***
Females	-0.02 (-0.07, 0.03)	0.4	0.01 -0.03, 0.06)	0.5	-0.02 (-0.06, 0.03)	0.5	-0.05 (-0.1, 0.01)	0.03	0.09
Distension (mm)									
DHA									
Model 1[*]	0.01 (-0.01, 0.02)	0.5	0 (0.02, 0.02)	1	-0.004 (-0.02, 0.01)	0.7	0.01 (-0.01, 0.03)	0.4	0.8
Males	0.02 (-0.01, 0.05)	0.2	-0.002 (-0.03, 0.03)	0.9	-0.01 (-0.04, 0.01)	0.3	0.01 (-0.01, 0.04)	0.3	0.9
Females	-0.002 (-0.02, 0.02)	0.9	-0.001 (-0.02, 0.02)	0.9	0.002 (-0.02, 0.02)	0.8	0.002 (-0.02, 0.02)	0.9	0.7
Model 2[†]	0.003 (-0.02, 0.02)	0.8	-0.001 (-0.02, 0.02)	0.9	-0.004 (-0.02, 0.01)	0.7	0.007 (-0.01, 0.02)	0.5	0.7
Males	-0.02 (-0.02, 0.05)	0.4	-0.004 (-0.04, 0.03)	0.8	-0.01 (-0.04, 0.02)	0.3	-0.02 (-0.02, 0.05)	0.3	0.9

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Females	-0.004 (-0.03, 0.02)	0.7	0 (-0.02, 0.02)	1	0.006 (-0.1, 0.03)	0.6	0.004 (-0.02, 0.03)	0.7	0.4
EPA									
Model 1[*]	0.01 (-0.01, 0.03)		0.001 (-0.02, 0.02)	0.9	0 (-0.02, 0.02)	1	0.02 (0.005, 0.04)	0.01	0.1
Males	0.001 (-0.03, 0.03)	1	-0.01 (-0.04, 0.02)	0.6	0.002 (-0.03, 0.03)	0.9	0.02 (-0.01, 0.05)	0.2	0.2
Females	0.02 (-0.004, 0.04)	0.1	.006 (-0.02, 0.03)	0.6	0.003 (-0.03, 0.02)	0.8	0.02 (0.001, 0.05)	0.04	0.3
Model 2[†]	0.01 (-0.01, 0.03)	0.3	0.002 (-0.02, 0.02)	0.9	-0.001 (-0.02, 0.02)	0.9	0.02 (0.003, 0.04)	0.02	0.1
Males	0.01 (-0.03, 0.04)	0.7	0 (-0.04, 0.04)	1	0.01 (-0.02, 0.05)	0.5	0.02 (-0.01, 0.06)	0.2	0.1
Females	0.01 (-0.008, 0.03)	0.2	0.008 (-0.01, 0.03)	0.5	0 (-0.02, 0.02)	1	0.02 (0.001, 0.05)	0.05	0.2
Right common carotid artery Distension (mm)									
DHA									
Model 1[*]	0 (-0.06, 0.06)	1	-0.02 (-0.08, 0.05)	0.6	0.02 (-0.04, 0.08)	0.6	0.02 (-0.04, 0.09)	0.5	0.4
Model 2[†]	-0.003 (-0.06, 0.06)	0.9	-0.05 (-0.1, 0.1)	0.1	-0.01 (-0.07, 0.05)	0.6	-0.01 (-0.07, 0.05)	0.8	0.8
EPA									
Model 1[*]	-0.01 (-0.08, 0.05)	0.7	-0.001 (-0.06, 0.06)	1	0.002 (-0.06, 0.07)	1	0.06 (-0.004, 0.1)	0.07	***0.05***
Model 2[†]	-0.01 (-0.07, 0.05)	0.7	0.004 (-0.06, 0.06)	0.9	-0.004 (-0.06, 0.06)	0.9	0.04 (-0.02, 0.1)	0.2	0.1

[1]For tables 9-5 – 9-9 quintile 1 is the lowest for fatty acid status (%RBCFA) (referent set at 0). Beta coefficient values[2] calculated as highest minus lowest (referent) quintile based on general linear models. ‡ *P* for trend across quintiles calculated with %RBCFA quintile modelled continuously. [*]Adjusted for baseline arterial diameter skin and room temperature. † Adjusted for baseline arterial diameter skin and room temperature age, sex, social class, physical activity, LDL cholesterol, triglycerides, WHR, fasting insulin and energy intake. Gender x fatty acid quintile interaction: *P* = 0.002 for FMD and 0.02 for EPA on brachial artery distension.

Table 9-6 Regression coefficients for CVD risk factors (obesity) according to quintiles[1] of fatty acids status

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Body Mass Index									
DHA									
Model 1[*;] **n=313**	-0.5 (-1.9, 0.9)	0.5	-0.9 (-2.2, 0.5)	0.2	-1.8 (-3.2, -0.5)	0.009	-1.4 (-2.7, 0.01)	***0.05***	***0.01***
Model 2 [†;] **n=297**	-0.08 (-1.3, 1.1)	0.9	-0.1 (-1.3, 1.0)	0.8	-1.03 (-2.2, 0.2)	0.09	-0.2 (-1.4, 1)	0.7	0.3
EPA									
Model 1[*]	-0.6 (-2, 0.7)	0.4	0.08 (-1.3, 1.4)	0.9	-1.3 (-2.7, 0.07)	0.06	-0.9 (-2.2, 0.5)	0.2	0.1
Model 2 [†]	-0.3 (-1.5, 0.9)	0.6	0.3 (-0.9, 1.5)	0.6	-0.5 (-1.7, 0.8)	0.5	0.4 (-0.9, 1.6)	0.6	0.6
Waist circumference, cm									
DHA									
Model 1[*]	-1.2 (-4.8, 2.3)	0.5	-2.6 (-6.1,0.8)	0.1	-5.1 (-8.6, -1.5)	0.005	-4.1 (-7.6, -6)	***0.02***	***0.003***
Model 2 [†]	-0.3 (-3, 2.5)	0.9	-0.5 (-3.2, 2.3)	0.7	-2.8 (-5.6, -0.2)	0.05	-1.3 (-4.1, 1.5)	0.4	0.1
EPA									
Model 1[*]	-2.9 (-6.5, 0.6)	0.1	-0.3 (-3.8, 3.3)	0.9	-2.3 (-6, 1.3)	0.2	-2.8 (-6.3, 0.8)	0.1	0.2
Model 2 [†]	-3.0 (-5.8, -0.2)	0.03	-0.3 (-2.7, 2.8)	1	-1.7 (-4.5, 1.2)	0.3	-1.1 (-3.9, 1.8)	0.5	0.9
Waist:Hip ratio									
DHA									
Model 1[*]	-0.1 (-0.04, 0.14)	0.4	-0.02 (-0.05, 0.002)	0.07	-0.03 (-0.6, -0.007)	0.01	-0.03 (-0.06, 0.008)	***0.009***	***0.002***
Model 2 *[†]	-0.002 (-0.02, 0.02)	0.9	-0.008 (-0.03, 0.009)	0.3	-0.02 (0.04, -0.001)	0.04	-0.01 (-0.03, 0.003)	0.1	***0.03***
EPA									
Model 1[*]	-0.02 (-0.04, 0.009)	0.2	-0.004 (-0.03, 0.02)	0.7	-0.01 (-0.04, 0.01)	0.3	-0.02 (-0.04, 0.005)	0.1	0.2
Model 2 [†]	-0.02 (-0.04, 0.001)	0.06	0 (-0.02, 0.02)	1	-0.02 (-0.04, 0.001)	0.06	-0.02 (-0.03, 0.002)	0.08	0.1

[*]Unadjusted,[†] Adjusted for age, sex and energy intake. Gender x fatty acid quintile interaction: $P > 0.07$ for all variables.

Table 9-7 Regression coefficients for CVD risk factors (blood pressure) according to quintiles[1] of fatty acid status

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Diastolic Blood Pressure (mm Hg)									
DHA									
Model 1[*]**: n=313**	1.3 (-1.5, 4.1)	0.4	0.7 (-2.0, -3.4)	0.6	-0.7 (-3.5, 2.0)	0.6	-2.5 (-5.3, 0.3)	0.08	***0.03***
Model 2[*†]**: n=393**	2.0 (-0.5, 4.5)	0.1	2.1 (-0.3, 4.6)	0.1	0.7 (-1.8, 3.2)	0.6	-1.1 (-3.6, 1.4)	0.4	0.2
EPA									
Model 1[*]	1.4 (-2.9, 2.6)	0.9	0.9 (-1.8, 3.6)	0.5	-1.4 (-4.3, 1.4)	0.3	-3 (-5.7, -0.2)	***0.04***	***0.02***
Model 2[*†]	0.8 (-1.7, 3.3)	0.5	1.2 (-1.2, 3.6)	0.3	-0.4 (-3.0, 2.1)	0.7	-1.3 (-3.8, 1.2)	0.3	0.2
Resting Heart Rate (beats per minute)									
DHA									
Model 1[*]	-2.0 (-1.7, 5.6)	0.3	1.9 (-1.7, 5.5)	0.3	1.8 (-1.8, 5.5)	0.3	-1.9 (-5.6, 1.7)	0.3	0.4
Model 2[*†]	2.0 (-1.5, 5.6)	0.3	2.0 (-1.5, 5.5)	0.3	0.5 (-3.1, 4.1)	0.8	-2.4 (-6.0, 1.2)	0.2	0.1
EPA									
Model 1[*]	1.3 (-2.3, 5.0)	0.5	2.3 (-1.3, 5.9)	0.2	-1.07 (-4.8, 2.7)	0.6	-3.5 (-7.3, 0.2)	0.06	***0.03***
Model 2[*†]	1.1 (-2.5, 4.6)	0.6	1.2 (-2.3, 4.7)	0.5	-1.0 (-4.7, 2.7)	0.6	-3.2 (-6.8, 0.5)	0.1	***0.05***

[*]Adjusted for skin and room temperature † Adjusted additionally for age, sex, social class, physical activity, LDL cholesterol, triglycerides, height, WHR, fasting insulin smoking practice and energy intake. Gender x RBCFA% quintile interaction for unadjusted models: *P* > 0.3 for all variables.

Table 9-8 Regression coefficients for CVD risk factors (fasting lipid concentrations) according to quintiles[1] of fatty acid status

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Total-cholesterol (mmol/L)									
DHA									
Model 1* **:n=317**	0.05 (-0.3, 0.3)	0.8	0.07 (-0.02, 0.4)	0.6	-0.2 (0.5, 0.1)	0.2	-0.3 (-0.5, 0.02)	0.06	***0.003***
Model 2 † **:n=299**	-0.03 (-0.3, 0.3)	0.9	0.1 (-0.2, 0.4)	0.4	-0.2 (-0.5, 0.1)	0.3	-0.2 (-0.5, 0.1)	0.2	0.1
EPA									
Model 1*	-0.2 (-0.1, 0.5)	0.3	-0.03 (-0.3, 0.3)	0.9	-0.04 (-0.3, 0.3)	0.8	-0.1 (-0.4, 0.2)	0.4	0.2
Model 2 †	0.2 (-0.1, 0.5)	0.2	0.004 (-0.3, 0.3)	1	0.04 (-0.3, 0.4)	0.8	-0.007 (-0.3, 0.3)	1	0.6
LDL cholesterol									
DHA									
Model 1*	0.1 (-0.4, 0.2)	0.4	-0.06 (-0.3, 0.2)	0.7	-0.2 (-0.5, 0.06)0.1	0.1	-0.3 (-0.6, -0.02)	***0.04***	***0.008***
Model 2 †	-0.06 (-0.3, 0.2)	0.7	0.05 (-0,2, 0.3)	0.7	-0.1 (-0.4, 0.2)	0.5	-0.2 (-0.4, 0.08)	0.3	0.2
EPA									
Model 1*	0.06 (-0.2, 0.3)	0.7	0.04 (-0.2, 0.3)	0.8	-0.1 (-0.4, 0.1)	0.3	-0.04 (-0.3, 0.2)	0.8	0.4
Model 2 †	0.1 (-0.1, 0.4)	0.3	0.04 (-0.2, 0.3)	0.7	-0.05 (-0.3, 0.2)	0.7	-0.05 (0-0.2, 0.3)	0.7	0.8
VLDL-cholesterol									
DHA									
Model 1*	-0.04 (-0.1, 0.06)	0.5	-0.1 (-0.2, -0.005)	0.04	-0.07 (-0.2, 0.03)	0.1	-0.1 (-0.2, 0)	***0.05***	***0.04***
Model 2 †	-0.03 (-0.1, 0.06)	0.5	-0.09 (-0.2, -0.002)	0.05	-0.05 (-0.1, 0.04)	0.3	-0.05 (-0.1, 0.04)	0.3	0.2
EPA									
Model 1*	-0.05 (-0.1, 0.04)	0.3	-0.06 (-0.2, 0.03)	0.2	-0.09 (-0.2, 0.01)	0.08	-0.1 (-0.2, -0.05)	0.004	0.07
Model 2 †	-0.05 (-0.1, 0.04)	0.3	-0.08 (-0.2, 0.01)	0.09	-0.06 (-0.2, 0.03)	0.2	-0.1 (-0.2, -0.004)	0.03	0.06
Triacylglycerol									

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
DHA									
Model 1[*]	-0.08 (-0.3, 0.1)	0.5	-0.2 (-0.4, -0.01)	0.04	-0.2 (-0.4, 0.06)	0.2	-0.2 (-0.4, 0)	***0.05***	***0.04***
Model 2 [†]	-0.07 (-0.3, 0.1)	0.5	-0.2 (-0.4, 0.03)	0.05	-0.1 (-0.3, 0.09)	0.3	-0.2 (-0.3, 0.08)	0.03	0.2
EPA									
Model 1[*]	-0.01 (-0.3, 0.1)	0.3	-0.1 (-0.3, -0.07)	0.2	-0.2 (-0.4, 0.03)	0.2	-0.3 (-0.5, -0.1)	***0.004***	***0.004***
Model 2 [†]	-0.1 (-0.3, 0.09)	0.3	-0.2 (-0.4, 0.03)	0.09	-0.1 (-0.3, 0.08)	0.2	-0.2 (-0.4, 0.009)	0.04	0.06

[*]Unadjusted,[†] Adjusted for age, sex, social class, physical activity, waist to hip ratio, mean arterial blood pressure, fasting insulin and energy intake. Gender x RBCFA% quintile interaction for unadjusted models $P > 0.1$ for all variables.

Table 9-9 Regression coefficients for CVD risk factors (Type 2 DM) according to quintiles[1] of fatty acid status

	Q2	P[2]	*Q3*	P[2]	*Q4*	P[2]	*Q5*	P[2]	p‡
Glucose (mmol/L)									
DHA									
Model 1* **:n=317**	-0.05 (-0.2, 0.1)	0.6	-0.2 (-0.3, 0.01)	0.03	-0.3 (-0.4, -0.09)	0.003	-0.3 (-0.5, -0.1)	***<0.001***	***<0.001***
Model 2 † **:n=299**	0.02 (-0.1, 0.2)	0.8	-0.08 (0.2, 0.07)	0.3	-0.2 (-0.3, 0.004)	0.06	-0.2 (-0.4, -0.06)	***0.004***	***0.001***
EPA									
Model 1*	-0.05 (-0.2, 0.1)	0.6	-0.006 (-0.2, 0.2)	0.9	0.05 (-0.1, 0.2)	0.6	-0.1 (-0.3, 0.05)	0.2	0.4
Model 2 †	0.03 (-0.2, 0.1)	0.8	0.02 (-0.2, 0.2)	0.9	0.08 (-0.1, 0.2)	0.4	-0.08 (-0.2, 0.09)	0.4	0.8
Insulin (pmol/L)									
DHA									
Model 1*	-6.7 (-16.2, 2.8)	0.2	-7.7 (-17. 1.6)	0.1	-5.1 (-14.6, 4.5)	0.3	-7.4 (-16.9, 2)	0.1	0.2
Model 2 †	-5.3 (-13, 2.5)	0.2	-6.1 (-13.8, 1.6)	0.1	-1.1 (-9, 6.8)	0.8	-1.9 (-9.7, 5.8)	0.6	0.1
EPA									
Model 1*	-4.8 (14.2, 4.5)	0.3	-1.1 (-10.4, 8.2)	0.8	-10 (-19.6, -0.4)	0.04	-11.7 (-21.1, -2.3)	***0.02***	***0.008***
Model 2 †	-0.8 (-8.6, 7.0)	0.8	2.7 (-5.1, 10.5)	0.5	-2.8 (-10.9, 8.3)	0.5	-3.8 (-11.8, 4.1)	0.3	0.3
HOMA									
DHA									
Model 1*	-0.3 (-0.6, 0.09)	0.1	-0.3 (-0.7, 00.2)	0.06	-0.3 (-0.6, 0.09)	0.1	-0.3 (-0.7, 0.02)	0.07	0.09
Model 2 †	-0.2 (-0.5, 0.1)	0.2	-0.2 (-0,5, 0.05)	0.1	-0.09 (-0.4, 0.2)	0.5	-0.09 (-0.4, 0.2)	0.6	0.8
EPA									
Model 1*	-0.1 (-0.5, 0.2)	0.4	-0.06 (-0.4, 0.3)	0.7	-0.3 (-0.7, 0.05)	0.09	-0.4 (-0.8, -0.03)	***0.03***	***0.02***
Model 2 †	-0.02 (-0.3, 0.3)	0.9	0.09 (-0.2, 0.4)	0.6	-0.05 (-0.4, 0.3)	0.7	-0.08 (-0.4, 0.2)	0.6	0.5

*Unadjusted,† Adjusted for age, sex, social class, physical activity, waist to hip ratio, mean arterial blood pressure, fasting LDL cholesterol, triglycerides and energy intake. Gender x RBCFA% quintile interaction for unadjusted models: $P > 0.2$ for all variables.

Chapter 10

Validation of a FFQ to estimate *n-3* LC-PUFA intake using red cell membrane fatty acids and multiple 24 hour dietary recalls

"True ideas are those that we can assimilate, validate, corroborate, and verify. False ideas are those that we cannot."
William James

10.1 Introduction

As discussed in **Chapter 4, Section 4.6**, the aim of dietary validation studies is to estimate the validity of methods used to assess dietary intake, by comparing associations between data derived from test instruments and a reference method that is accepted to accurately reflect true intake.[628] The validity of this approach relies on two assumptions: 1) that random errors between questionnaires and reference methods are independent and 2) random errors within reference methods are also random and independent. It is possible, however, that random errors of questionnaires and reference methods are positively correlated, leading to overestimation of validity. For example, both FFQs and 24-hour recall methods rely on the participant's ability to recall and describe foods eaten accurately and both may share potential sources of error. Conversely, covariance between replicate reference measurements (i.e. multiple day recordings) can lead to underestimation of the validity of a questionnaire under test. For example, failure to record foods that have actually been consumed on a day-to-day basis or a true change in dietary behaviour on recording days may result in underestimation of attenuation in the reference method. This, in turn, would result in underestimation of the test reference method's accuracy. Therefore, it is questionable whether random errors of reference dietary assessment methods are independent of those attached to FFQs. Under free living conditions, true intake is unknown and assessments of validity are relative. Therefore, studies that use reference dietary assessment methods to assess the validity of new questionnaires are more correctly termed comparison studies.

Biochemical markers of nutrient intake, in which sources of random error differ from those of dietary assessment methods, are increasingly used in dietary

validity studies to minimise risks of correlated error. Individual fatty acid concentrations in erythrocyte membranes are useful biomarkers of fatty acid intake as they provide an objective marker and do not rely on participants' abilities to recall intake or their compliance with methods recording consumption. Red cell membrane fatty acid concentrations are also reported to reflect long-term intake and are therefore better markers of habitual diet **(Chapter 4, Section 4.6.1)**. Furthermore, they are not prone to the same measurement errors as dietary assessment methods including inaccuracies in portion size estimation and food composition database values.

Although FFQs are available which have been validated in the UK population,[530, 552] none is available that has been assessed objectively for dietary intake of *n-3* LC-PUFA. FFQs that cover a wide range of foods can be long and complicated, imposing a considerable burden on participants and therefore compromising reliability of the method. Shortening the questionnaire, to group foods together, can lead to a loss of detailed information and underestimation of intake of specific nutrients. However, where measurement of a single or few nutrients is required a shorter FFQ focussing on those nutrients may be more practical.

Outside the UK, some attempts have been made to develop FFQs for measuring either dietary fish[556, 559, 561, 629] or fatty acid intake[553, 555, 557, 558, 560, 565, 630-632] but to our knowledge no validated tool is available in the UK where the population and dietary habits differ from those in countries where previous questionnaires have been validated. Therefore this chapter investigates the association between fish intake from a newly developed questionnaire and biomarker status.

A major aim of this PhD was to develop a FFQ designed to estimate habitual intake of *n-3* LC-PUFA in the UK population (FishFQ). Dietary intake data collected by FFQs and multiple 24-hour dietary recalls as part of the RCT were analysed and compared. Red blood cell membranes were analysed for their fatty acid content to provide an objective assessment of *n-3* LC-PUFA status. If shown to be valid, the FishFQ will be useful in both clinical and research settings where a validated dietary assessment tool is urgently required.

10.2 Subjects & Methods

A total of 324 healthy young adults took part in the study. Background diet was assessed using the EPIC FFQ. 323 subjects completed the FFQ at baseline and one hundred and twenty five subjects took part in the validation study which required: 1) completion of a specifically designed FishFQ, 2) completion of multiple 24-hour records of dietary recall and 3) a fasting blood sample for measurement of *n-3* fatty acid erythrocyte membrane concentration. Ninety-one subjects completed both dietary assessment methods required for the validation study and provided a venous blood sample for analysis of red cell membrane fatty acids. Demographic variables and information relating to lifestyle practices, health behaviours and medical history were collected as part of the main RCT. Primary and secondary outcomes including, vascular, haematological anthropometric and body composition measurements were carried out by trained researchers and erythrocyte membrane fatty acid concentrations were measured. Full details of study procedures can be found in **Chapter 5**.

Fatty acid intakes from the EPIC and Fish FFQs were calculated using the microdiet nutritional analysis programme, version 2.8.8 (Downlee systems, England). Nutrient intake was estimated using data from the 4th, 5th and 6th editions of McCance and Widdowson's "The Composition of Foods", Royal Society of Chemistry, Ministry of Agriculture Fisheries and Foods[520], and its supplements. The 5th edition includes the seventh supplement that provides detailed fatty acid data.[367] 24-hour recalls were analysed as described in **Chapter 6, Section 6.4.4**. Energy intake based on 24-hour recall was used to identify subjects with extremes indicating implausible energy intakes (>16 800kj/d and <3360kj/d for males and >14700 kj/d and <2100 kj/d for females.[633] No individual was excluded on this basis. Fatty acid intakes are from food alone (exclusion criteria for participation in the RCT included use of *n-3* supplements, **(Chapter 5, Section 5.2.3)**.

10.3 Results

Subject characteristics at study baseline are reported in **Table 10-1.** There were no significant differences between participants and non-participants for most

variables with the exception of smoking and glucose concentration. A greater percentage of non participants were current smokers compared with participants (*n* = 104/233, 45% vs. *n* = 24/91, 26% for non- and participants respectively [*P* < 0.002]). Fasting blood glucose was 6% higher in participants (5 vs. 4.7mmol/L in participants and non-participants respectively [*P* < 0.001]).

10.3.1 Agreement Between Fish and EPIC FFQs

After recruitment of the first 100 subjects agreement between the new questionnaire and the EPIC FFQ was assessed using the Bland and Altman technique.[607] Estimates of nutrient intake were based on standard portion sizes. Eighty eight out of one hundred subjects completed both the EPIC and FishFQ at baseline. The FishFQ gave higher estimates of EPA and DHA intake compared with the EPIC FFQ. The two methods were significantly correlated (*r* = 0.31, *P* < 0.01) but agreement between them was poor.

For DHA mean (SD) intake using the EPIC FFQ was 0.15g/d (0.19) and using the new FFQ 0.79g/d (0.48). The mean (SD) of the differences between the two methods (bias) was -0.63g/d (0.46). Limits of agreement, calculated as 2 x SD were (-1.55 to 0.29) **(Figure 10-1)**.

With respect to EPA the mean (SD) intake based on EPIC was 0.11g/d (0.14) and 0.68g/d (0.47) using the new FFQ, mean (SD) bias was -0.57g/d (0.48) and 95% limits of agreement -1.52 to 0.39 **(Figure 10-2)**. This represents poor agreement and suggests that the two methods cannot be used interchangeably.

From this analysis it is not possible to conclude which method provides the most accurate assessment. Compared with the EPIC FFQ, the Fish FFQ gave 4-5 fold higher mean estimates of *n-3* LC PUFA intake possibly because the broader (EPIC) questionnaire does not separate *n-3* LC PUFA foods within food groups. However, it is also possible that *n-3* LC PUFA intake was overestimated using the new questionnaire. Further research to test the ability of the new food frequency questionnaires to assess *n-3* LC PUFA intake against biomarkers would help to elucidate this.

Figure 10-1 Bland and Altman Plot of mean versus difference in DHA intake (g/day) estimated using EPIC and FishFQ (n=88)

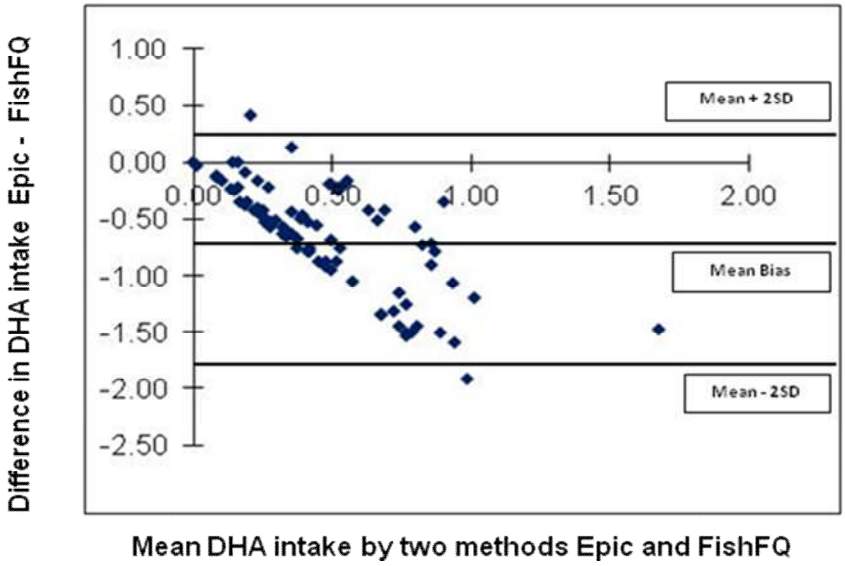
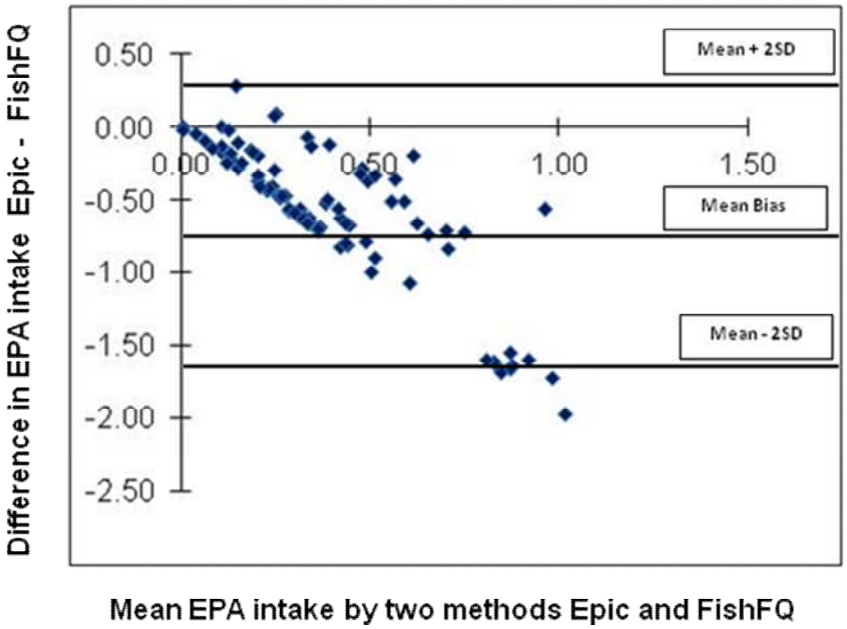


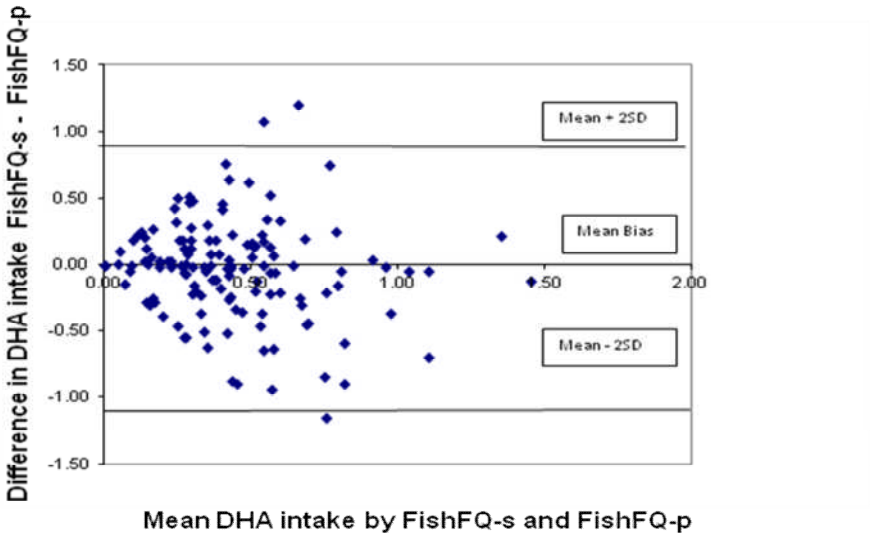
Figure 10-2 Bland and Altman Plot of mean versus difference in EPA intake (g/day) estimated using EPIC and FishFQ (n=88)



10.3.2 Standard versus estimated portion size

Comparisons between estimates of fish intake using the EPIC and Fish FFQs based on standard portion sizes and amounts depicted in photographs were carried out in a subset of 138 subjects who provided information on portion size using the photographic portion size atlas. Estimates of *n-3* LC PUFA foods were higher when standard portions were applied to calculate daily amounts consumed in grams compared with portions based on estimates from photographs. The two methods were significantly correlated (r = *0*.31, P = 0.01) and agreement between them (analysed using the Bland and Altman (1986) method) was reasonable. For DHA mean (SD) intake using standard portions was 0.45g/d (0.34) and using portions depicted in photographs 0.41g/d (0.29) The mean (SD) of the differences between the two methods (bias) was -0.04g/d (0.37). Limits of agreement were quite wide at (-0.74 to 0.74g/d) suggesting a high degree of variation for some subjects **(Figure 10-3)**.

Figure 10-3 Bland and Altman Plot of mean versus difference in DHA intake (g/day) estimated using FishFQ with standard (FishFQ-s) and reported (FishFQ-p) portion sizes



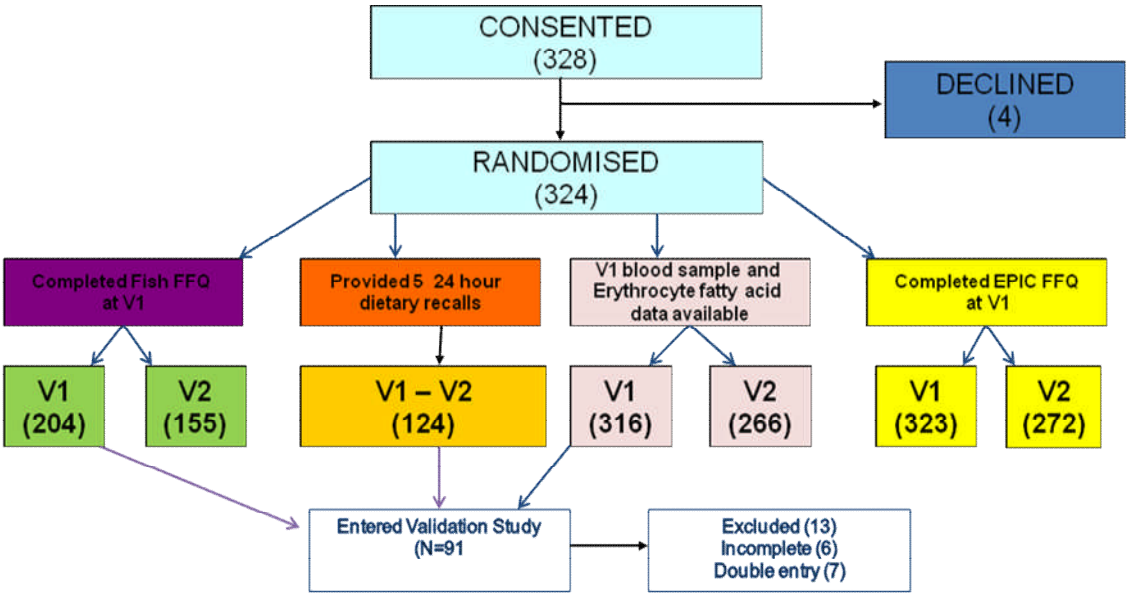
This relatively small bias means that the two methods can be used interchangeably. Therefore, in view of extra costs and greater burden on study participants and researchers with the provision and use of a photographic atlas it

was decided that validation of the FishFQ would be based on standard portion size estimations.

10.3.3 Validation Studies

Ninety-one subjects completed the FishFQ and provided 5 24-hour recalls of dietary intake. Thirteen subjects were excluded for various reasons including: inadequate completion of the FishFQ (*n* = 6) and implausible data (*n* = 7) (e.g. ticked more than one frequency category in a single line entry). Data were considered implausible where outliers introduced positive skewness or kurtosis with significant effects during exploratory analyses. Therefore, a total of seventy eight subjects were included in the validation analyses reported below (**Figure 10-4**).

Figure 10-4 Participant flow through dietary assessment protocol



10.3.3.1 Correlations of FFQs with biomarkers of LC-PUFA intake

Pearson's correlation coefficients between the EPIC and Fish FFQs, are shown in **Table 10-2.** Correlations between the FishFQ and the biomarker were moderate to high (r = 0.34, 0.38 and 0.43 for DHA, EPA and DHA+EPA respectively [P < 0.01]). In contrast most correlations between measurements of *n-3* LC-PUFA intake using the EPIC FFQ and the biomarker were lower ranging from r = 0.22 for DHA [P < 0.05] to r = 0.40 [P < 0.01] for EPA and r = 0.27 [P < 0.01] for combined DHA and EPA. This suggests that overall the new FishFQ provides a more accurate assessment of *n-3* LC-PUFA intake than the EPIC FFQ. Adjustments for age, sex and energy intake did not consistently improve correlations.

10.3.3.2 Validation of FishFQ using the method of Triads

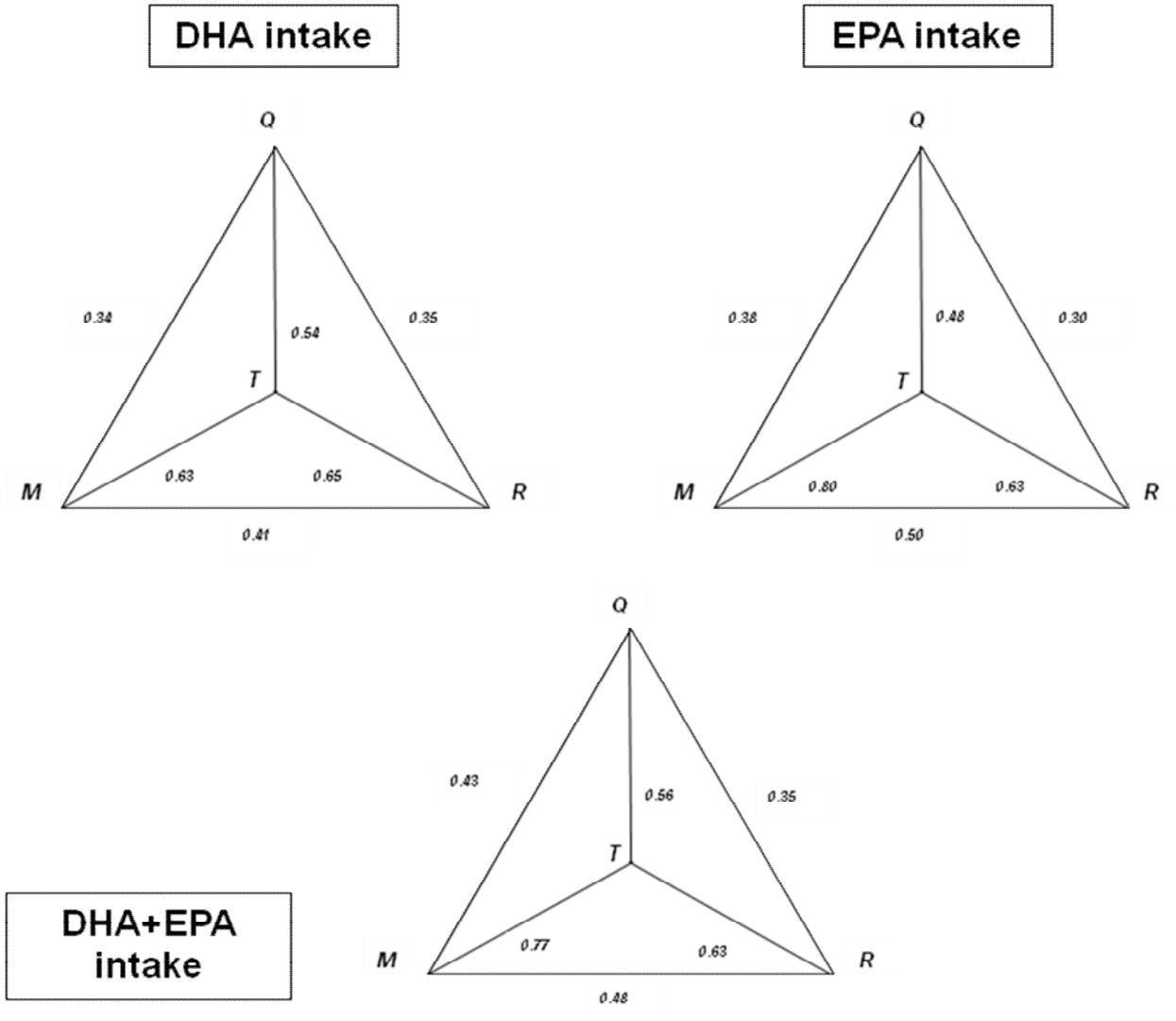
Because adjustments for age, sex and energy intake did not consistently improve correlations, unadjusted correlation coefficients between the two dietary assessments and the biomarkers of fatty acid status were used to calculate validity coefficients. Relative validity of the FishFQ was assessed using the method of triads which uses triangular comparisons between a dietary assessment method under test (FishFQ), a reference method (multiple 24-hour dietary recalls) and a biochemical marker (erythrocyte membrane fatty acids (RBC FA) to compute validity coefficients **(Chapter 6, Section 6.5.4.1 and Figure 6-2).**

Triangular validation methodology assumes that correlations between the three measurements reflect a linear relationship of each method with the true intake **(Figure 10-5)**. The correlation coefficient between the test instrument (FishFQ) and the biomarker provides a lower limit for estimation of validity. The method of triads improves the estimation of validity and provides an upper limit. Equations for calculation of validity estimations used in the present validation study are reported in **Appendix 4-1**.

The 95% confidence intervals for the validity coefficient were estimated using bootstrap sampling where 200 samples of equal size (n = 78) were obtained

with replacement from the study participants. Analyses were carried out in PASW, version 18 (SPSS Inc., Chicago, IL, USA). Validity coefficients for the FishFQ were 0.54 for DHA, 0.48 for EPA and 0.56 for combined EPA/DHA. Validity coefficients for the 24 hour recall method were generally higher than the FishFQ and the biomarker had the greatest validity **(Table 10-4)**

Figure 10-5 Sample correlations and validity coefficients for triangular comparisons between FFQ (*Q*), 24-h recall (*R*) and biomarker (*M*). T represents the hypothetical true intake



10.3.4 Reliability

Reproducibility of the EPIC and FishFQs was assessed by comparing values obtained at study baseline with those collected after the 4-month intervention period. 155 subjects completed the final version of the FishFQ at study baseline and 4 months later following the intervention period. 272 subjects completed the EPIC FFQ at both time points. Pearson's correlation coefficients between the two administrations of the EPIC and FishFQ are shown in **Tables 10-5 and 10-6.**

10.3.5 Agreement According to Bland and Altman Analysis

Agreement was further assessed using the Bland and Altman method as described in **Chapter 6, Section 6.3.5.1**. Both questionnaires showed good agreement. Mean bias for DHA was 6% and 5% for the Fish and EPIC FFQ respectively **(Table 10-7 and 10-8, Appendix 4-3)**. Limits of agreement were wide (± 0.96g/d and ± 0.38g/d for the Fish and EPIC FFQ respectively **(Table 10-7 and 10-8)**. The small bias in DHA intake, estimated at two time points, demonstrates good agreement at the group level and suggests that both questionnaires provide acceptable estimates of intake in this population. However, the wide limits of agreement suggest this good agreement does not extend to individuals.

10.3.6 Classification to Population Distribution

Agreement between the Fish and EPIC FFQ and biomarkers of fatty acid status was assessed in 78 participants for whom complete sets of data were available (completed both FFQs and provided a blood sample at baseline and post-intervention). The proportions of individuals classified in the same third of the distribution (exact agreement) and into extreme thirds of the distribution (gross misclassification) were calculated. Using the FishFQ exact agreement with biomarker status was reported in 57% of subjects for DHA and 56% for EPA at study baseline. Classification according to EPIC was less accurate (44% for DHA and 46% for EPA) **(Table 10-8)**. Exact agreement between intake assessments and fatty acid status according to biomarkers improved for the

Fish and EPIC FFQ post intervention. Using the FishFQ exact agreement with biomarker status was reported in 58% of subjects for DHA and 60% for EPA. Exact agreement between intake assessment using the EPIC FFQ and fatty acid status according to biomarkers was 55% for DHA and 47% for EPA) **(Table 10-8)**.

These findings suggest the Fish and EPIC FFQ can accurately place individuals according to their estimated DHA and EPA intake. Furthermore, the superior agreement found between the FishFQ and biomarkers suggests the new questionnaire may offer a less costly and time consuming method for ranking individuals according to DHA and EPA intake.

Table 10-1 Characteristics at baseline comparing participants in validation study with non participants

	Participants	Non Participants	P
n	**91**	**233**	
Age, y	27.6 (4.8)	27.9 (4.7)	0.4
[1]Male gender, % , n	38 (35)	35 (85)	0.7
[1]Non-manual, %, n	55 (50)	58 (135)	0.4
[1]Current smoker, % , n	26 (24)	45 (104)	***0.002***
Body mass index, kg/m^2	23.7 (4.3)	23.5 (3)	0.6
% fat (skinfolds)	23.6 (5.5)	24.1 (5.5)	0.5
Sum skinfolds, mm	53.7 (19.0)	50.5 (20.9)	0.2
Waist circumference, cm	77.4 (10.4)	77.3 (10.0)	1.0
Waist:Hip ratio	0.8 (0.07)	0.78 (0.07)	0.5
Blood pressure, mm Hg			
Systolic	112 (10)	111 (11)	0.8
Diastolic	66 (7)	67 (8)	0.4
Mean Arterial	84 (8.)	84 (9)	0.9
Pulse Pressure	46.0 (6)	45.0 (6)	0.2
Heart rate, beats/min	66 (10)	66 (11)	0.7
Total cholesterol, mmol/L	4.2 (0.8)	4.3(0.8)	0.4
LDL cholesterol, mmol/L	2.4 (0.8)	2.4 (0.8)	0.9
HDL cholesterol, mmol/L	1.4 (0.4)	1.5 (0.3)	0.4
VLDL cholesterol	0.5 (0.3)	0.4 (0.3)	0.9
Total:HDL cholesterol ratio	3.2 (1.1)	3.1 (0.1)	0.6
LDL:HDL cholesterol ratio	1.8 (0.9)	1.7 (0.8)	0.4
Triglyceride, mmol/L	1.0 (0.6)	1.0 (0.6)	0.9
Glucose, mmol/L	5.0 (0.5)	4.7 (0.5)	***<0.001***
Insulin, pmol/L	35.8 (42.0)	29.7 (17.6)	0.07
Insulin resistance (HOMA)	1.2 (1.6)	0.89 (0.6)	0.1
CRP, mg/L	1.8 (3.2)	1.7 (3.4)	0.67
Cotinine, ng/ml	22.6 (73.2)	25.3 (79.3)	0.8

Data are mean (SD) except: [1] % (n) <5% loss of n for some variables.

Table 10-2 *N-3* Fatty Acid Intake and correlation coefficients between EPIC and Fish FFQ and erythrocyte *n-3* fatty Acids. (*n* = 78)

	Intake, g/d, mean (sd)		Pearson's correlation coefficients			
			Unadjusted		Adjusted**	
Fatty Acids	Fish FQ	EPIC FFQ	FishFQ vs. Biomarker	EPIC FFQ vs Biomarker	FishFQ vs Biomarker	EPIC FFQ vs Biomarker
DHA	0.41 (0.2)	0.21 (0.2)	0.34	0.22*	0.33	0.20*
EPA[1]	0.47 (0.3)	0.26 (0.2)	0.38	0.40	0.35	0.40
EPA + DHA	0.82 (0.5)	0.47 (0.5)	0.43	0.27	0.37	0.28

All data except[1] were $\log_e$ transformed before analyses. All correlations significant at 0.01 level (two tailed) except * significant at 0.05 level. ** Adjusted for age, sex and energy intake in regression analyses.

Table 10-3 Pearson's correlation coefficients (95% confidence intervals) between FishFQ, 24-Hour recall and erythrocyte *n-3* fatty acids(*n* = 78)

	FishFFQ vs. 24 Hour Recall	24 Hour Recall vs. Biomarker	FishFFQ vs. Biomarker
DHA	0.35 (0.14, 0.52)	0.41 (0.20, 0.60)	0.34 (0.08, 0.57)
[1]EPA	0.30 (0.09, 0.52)	0.50 (0.26, 0.66)	0.38 (0.20, 0.58)
EPA+DHA	0.35 (0.16, 0.51)	0.48 (0.21, 0.68)	0.43 (0.14, 0.63)

Data are $\log_e$ transformed except[1] and presented as correlation coefficient (95% confidence interval). All correlations significant at 0.01 level (two tailed).

Table 10-4 Validity coefficient of the FishFQ, 24-Hour Recalls (24-HR) and biomarker of *n-3* LC-PUFA intake as calculated by the method of triads and the 95% CI (*n* = 78)

	Validity coefficients						Range of Validity coefficients†		
	***P*QT**	**95% CI**	***P*RT**	**95% CI**	***P*MT**	**95% CI**	***P*QT**	***P*RT**	***P*MT**
DHA	0.54	(0.22,0.92)	0.65	(0.32, 1.28)	0.63	(0.29, 1.08)	0.34 – 0.54	0.41 – 0.65	0.34 – 0.63
EPA	0.48	(0.26, 0.76)	0.63	(0.33, 1.00)	0.80	(0.48, 1.44)	0.38 – 0.48	0.50 – 0.63	0.38 – 0.80
EPDHA	0.56	(0.32, 0.88)	0.63	0.37, 1.04)	0.77	0.37, 1.23)	0.43– 0.56	0.48 – 0.63	0.43 – 0.77

† The lower limit for the FishFQ and the biomarker is the correlation between the Fish FQ and the biomarker, and the lower limit for the 24-HR is the correlation between the biomarker and the 24-HR. The upper limit is calculated by the method of triads.
pQT, validity coefficient of the FishFQ, pRT, validity coefficient of the 24-H recall, pMT, validity coefficient of the biomarker.
All data natural log transformed. 5% confidence intervals computed from 200 Bootstrap samples

Table 10-5 Pearson's correlation coefficients for the FishFQ administered on two occasions at 4 month interval (n=155)

	Fish FQ Visit 1			Fish FQ Visit 2			*R*	Difference (% mean intake)
Fatty Acid	**Mean**	**sd**	**Range**	**Mean**	**sd**	**Range**		
DHA (g/day)	0.54	0.66	0 – 4.23	0.51	0.55	0 – 4.59	0.74	6%
EPA (g/day)	0.57	0.48	0 – 2.77	0.50	0.46	0 – 3.56	0.55	15%

Table 10-6 Pearson's correlation coefficients for the EPIC FFQ administered on two occasions at 4 month interval (n= 272)

	EPIC FFQ Visit 1			EPIC FFQ Visit 2			*R*	Difference (% mean intake)
Fatty Acid	**Mean**	**sd**	**Range**	**Mean**	**sd**	**Range**		
DHA (g/day)	0.22	0.21	0.00 – 1.49	0.20	0.21	0.00 – 1.61	0.64	5%
EPA (g/day)	0.29	0.36	0.00 – 4.52	0.29	0.36	0.00 – 3.52	0.32	3%

Table 10-7 The relative bias and limits of agreement for measurement of DHA and EPA estimated by Fish and EPIC FFQ at visit 1 and visit 2

	FishFQ		EPIC FFQ	
	DHA (g/day)	EPA (g/day)	DHA (g/day)	EPA (g/day)
Bias	0.03	0.08	0.01	0.01
Limits of agreement	± 0.96	± 0.91	± 0.38	± 0.83
Bias as percentage mean intake	6%	15%	5%	3%

Table 10-8 Agreement between fatty acid intakes estimated by Fish and EPIC FFQ and status according to biomarkers

Fatty Acid	Baseline								Post-intervention							
	Fish FQ and biomarker				EPIC FFQ and biomarker				Fish FQ and biomarker				EPIC FFQ and biomarker			
	Exact (%)	GM (%)	*k*	*P*	Exact (%)	GM (%)	*k*	*P*	Exact (%)	GM (%)	*k*	*P*	Exact (%)	GM (%)	*k*	*P*
DHA	***57***	8	*0.3*	*<0.01*	***44***	12	*0.3*	*<0.01*	***58***	7	*0.4*	*<0.001*	***55***	2	*0.3*	*<0.001*
EPA	***56***	11	*0.2*	*0.03*	***46***	16	*0.2*	*0.01*	***60***	9	*0.4*	*<0.001*	***47***	19	*0.2*	*0.006*

All correlations significant at 0.01 level. DHA= Docosahexaenoic acid, EPA=Eicosapentaenoic Acid. GM = gross misclassification [1]Fatty acids expressed as % total erythrocyte membrane fatty acids. †<5% missing data for some variables. k = kappa value

10.4 Summary

The new questionnaire estimated dietary intake of *n-3* LC-PUFA well compared with results from previous studies. Correlation with the reference dietary assessment method was moderate and comparable to that found in other studies for DHA and EPA. More rigorous evaluation using an independent biomarker of *n-3* LC-PUFA status suggests validity may be even higher.

The questionnaire also demonstrated good reproducibility indicating a high level of precision. Importantly, agreement between assessments of *n-3* LC-PUFA intake using the new questionnaire and fatty acid status according to the biomarker was also good and the questionnaire was able to place more than 50% of participants into the same thirds of the distribution when intake was assessed by FFQ and fatty acid status evaluated through red cell membrane concentration. Only about 10% of participants were misclassified. This compares closely with other studies where exact agreement was also reported for about 50% of participants.[555, 632]

10.5 Discussion of Results

Less than 20 studies have validated dietary methods for assessment of *n-3* fatty acids using biomarkers and a universally accepted marker of *n-3* LC-PUFA status has yet to be identified.[634] Biomarkers considered to produce reliable estimates include subcutaneous adipose tissue, plasma and erythrocyte fatty acid concentrations. Studies comparing effects of supplementation with fatty acids on different compartments have concluded that erythrocyte membrane concentration is a better index for monitoring long-term intake of *n-3* fatty acids, whereas plasma phospholipids are more sensitive to short-term changes.[635]

Fifteen published reports of validation studies comparing FFQs with biomarkers of fatty acid status are currently available.[634] Four of these have assessed validity against RBC FA concentrations.[632, 636-638] Eight studies used plasma fatty acids or phospholipids.[545, 546, 553, 555, 556, 558, 638, 639] Only two studies compared intake assessed using FFQ with subcutaneous fat concentrations.[542, 640] Correlation

coefficients between biomarkers and fatty acid intakes estimated from FFQs are similar but vary according to the chosen biomarker. Studies that have used RBC FA concentrations as a biomarker report unadjusted correlation coefficients in the range of 0.23-0.36 for EPA and 0.19-0.54 for DHA. Where plasma fatty acids are used correlation coefficients are generally higher and range from 0.25-0.56. Correlations with adipocyte fatty acids are low ranging from 0.15 to 0.32.

Although it is not clear why biomarker correlations vary, fatty acids in plasma may be present in many forms (for example as part of cholesterol esters, triglycerides or phospholipids) and are affected by dietary intake and therefore more variable. Equally the more complex array of fatty acids in adipose tissue compared with erythrocyte membranes means that assays are more prone to error.[544]

Many FFQs are designed to capture usual (habitual) dietary intake and therefore collect information on a wide range of foods. For example in studies discussed above FFQs included between 66 and 360 foods. However, only two questionnaires were specifically designed to ask about foods high in *n-3* LCPUFA.[558, 632] Only one study specifically asking about *n-3* foods used RBC FA as the reference biomarker.[553] Most studies used correlation analysis to estimate validity and only one study applied the methods of triads to provide an upper limit.[555]

Sullivan and colleagues (2006) reported correlation coefficients between DHA and EPA intake, assessed with a FFQ specifically designed to measure *n-3* LCPUFA intake, and RBC FA concentrations of $r = 0.39$ and 0.4 respectively ($n = 53$). This agrees closely with the findings of the present study that found similar correlations of $r = 0.34$ and $r = 0.38$ for DHA and EPA respectively.

The one other study identified that used the method of triads to validate a tool for dietary *n-3* assessment validated a generalized FFQ against weighed food records and plasma fatty acids.[555] This study reported validity coefficients for the FFQ of

0.62 and 0.54 for DHA and EPA respectively (*n* = 43). These coefficients are slightly higher, although still in the same range, than those found in the present study (0.54 and 0.48 for DHA and EPA respectively). The reason for this is unclear. One possibility is that McNaughton used weighed records as the reference dietary assessment and plasma fatty acid concentration as a biomarker. Weighed records provide a snapshot assessment of dietary intake and plasma fatty acids reflect recent rather than habitual intake. Therefore high correlation between these two methods may have improved the overall estimate of validity.

One possible drawback with the study of McNaughton is that FFQs are designed to assess habitual intake rather than to provide a snapshot of recent intake. Therefore comparison against weighed records and plasma fatty acids which both measure recent intake may not have been appropriate. This is supported by the fact that correlation coefficients between the FFQ and biomarkers reported by McNaughton were lower than in the present study (*r* = 0.32 and *r* = 0.21 for DHA and EPA respectively compared with *r* = 0.34 and 0.38 in the present study).

Our study shows that the FishFQ can be used in both clinical and research settings to identify individuals with low, medium or high intakes of *n-3* LC-PUFA. This is important because reliable estimates of intake are needed to interpret research findings correctly and underpin public health messages. In clinical practice, accurate assessment of intake is needed to inform dietetic interventions and identify individuals at increased risk of nutritional deficiencies. The new FFQ can also be used to classify individuals at increased risk. For example, where relationships between *n-3* fatty acid consumption and specific diseases have been identified including cardiovascular disease[641] and prostatic cancer[637] The findings of this study have recently been presented at the Summer Meeting of the Nutrition Society and the abstract has been accepted for publication in the Proceedings of the Nutrition Society **(Appendix 4-2)**.

Chapter 11

Overall Discussion and Conclusions

Life is the art of drawing sufficient conclusions from insufficient premises.
Samuel Butler

11.1 Summary of Findings

In **Chapter 5, Section 5.1.1**, the main hypotheses for the RCT and epidemiological dietary study were set out. The aim of the RCT was to test the hypothesis that *n*-3 PUFA supplementation improves vascular function and CVD risk factors in healthy young adults. The epidemiological study tested the hypotheses that dietary patterns and components within them (specifically *n-3* LC-PUFA) are associated with vascular structure and function and CVD risk factors.

No validated tool for assessment of *n-3* LC-PUFA was available in the UK. Therefore, I designed and developed a new food frequency specifically to assess *n*-3 LCPUFA intake in this population of young healthy adults. I hypothesized that this new FFQ would demonstrate acceptable validity when compared against a reference dietary assessment method (multiple 24-hour dietary recalls) and biomarkers of *n-3* LC-PUFA intake.

In **Chapter 7** a RCT investigating the impact of DHA supplementation on FMD and surrogate markers of atherosclerosis in healthy young people found a small but negative effect on FMD in men. However, DHA supplementation had beneficial effects on CVD risk factors. Blood pressure was reduced in men randomized to receive 1.6g/d DHA for 4 months and VLDL and TG were reduced in men and women.

In **Chapter 8** I identified 3 dietary patterns in the study participants. In univariate analyses these dietary patterns were associated with measures of vascular structure (carotid artery intima media thickness and brachial artery distensibility)

and CVD risk factors including BP, heart rate, serum lipid concentrations, fasting insulin concentration (a marker of T2DM risk) and CRP (a marker of inflammation and endothelial activation). After adjustment for risk factors known to influence vascular health and CVD in multivariate models many relationships were attenuated and no longer significant. However, independent associations of dietary patterns with CCA-IMT, brachial artery distensibility, BP, heart rate, VLDL and TG concentrations remained.

In secondary analyses associations of dietary patterns with vascular structure and CVD risk factors differed according to gender. Associations of dietary patterns with CCA-IMT and brachial artery distensibility were confined to women. There were no significant gender interactions with dietary patterns on CVD risk factors except for CRP where the relationship was again confined to women. However, this association was no longer significant after adjustment for other CVD risk factors suggesting that associations of diet with endothelial inflammation and activation are mediated through other CVD risk factors.

Dietary patterns were not directly associated with endothelial function assessed through measurement of FMD. However, many CVD risk factors showed moderate to strong correlations with vascular structure. In particular, BP was strongly related to CCA distensibility. This suggests that associations of habitual diet with FMD are likely to operate through other CVD risk factors including increased BP, heart rate, and VLDL and TG concentrations.

In Chapter 9 I found that fatty acid status, assessed by red cell membrane concentration, was associated with FMD in women. Unexpectedly, as DHA status increased FMD decreased. Relationships were also found between fatty acid status and CVD risk factors including obesity, BP, heart rate, serum lipid concentrations and fasting glucose and insulin. In univariate analyses higher DHA status was associated with lower BMI, WC, WHR, BP, TC, LDL, VLDL, TG and fasting blood glucose (FBG). After adjustment for potential confounding factors

many associations were attenuated and no longer significant. However, relationships between higher DHA status and WHR and FBG persisted in fully adjusted models **(Tables 9-6 and 9-9).**

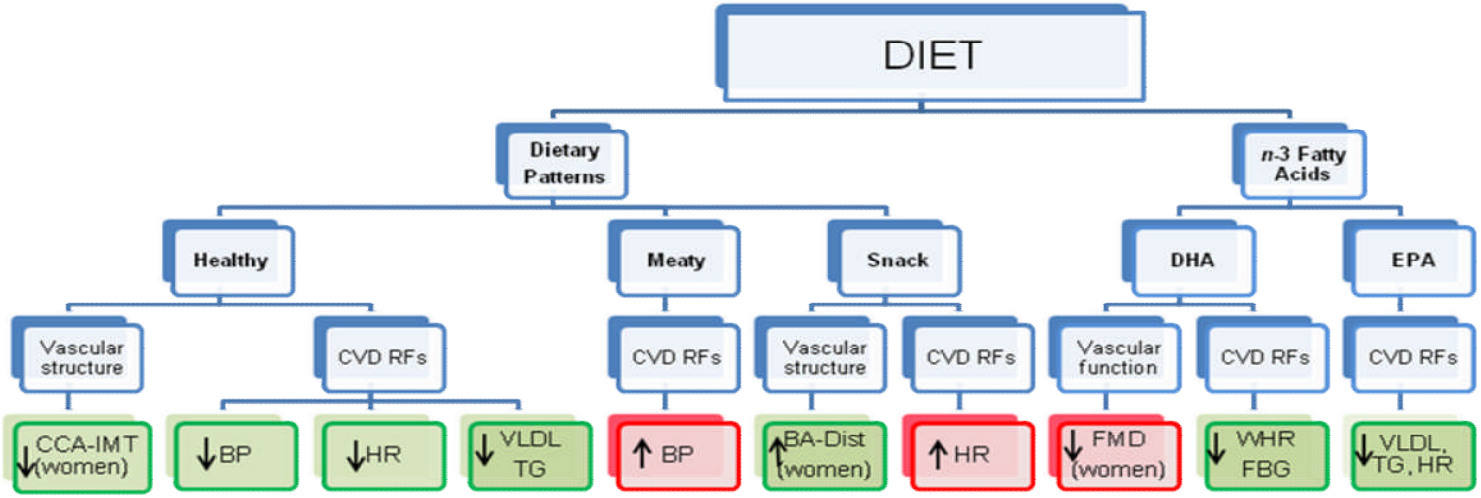
Higher EPA status was associated with lower diastolic blood pressure, VLDL, TG and resting heart rate. After adjustment for confounding factors, a trend remained for the association of EPA status with VLDL, TG and HR. This suggests that *n-3* fatty acids may be dietary components within a healthy dietary pattern that are associated with benefits for the lipid profile and reductions in CVD risk.

In **Chapter 10** the new FishFQ was found to be a valid and reliable tool for assessment of *n-3* LC-PUFA intake in this population of young UK adults. The FishFQ demonstrated acceptable validity when compared with multiple 24-hour dietary recalls and biomarkers of fatty acid status using triangular comparisons. The FishFQ was also able to correctly classify individuals as low, intermediate or high *n*-3 LC-PUFA consumers. More than half were placed in the same thirds of the population distribution according to intake assessment and fatty acid status.

In summary, the RCT found that DHA directly affected FMD negatively in men. Benefits of DHA supplementation were found for blood pressure but these were confined to men. Significant reductions in triglyceride and VLDL concentrations were found in women and men.

In the epidemiolgoical study, dietary patterns were associated with vascular structure in women. No direct associations of dietary patterns with FMD were apparent. However, dietary patterns were associated with a range of CVD risk factors. Relationships between dietary patterns and BP, VLDL and TG remained after adjustment for confounding factors demonstrating independent associations of dietary patterns with these risk factors. Findings from the RCT and epidemiological study are summarised in **Figure 11-1 and 11-2** and discussed in **Section 11.2** below.

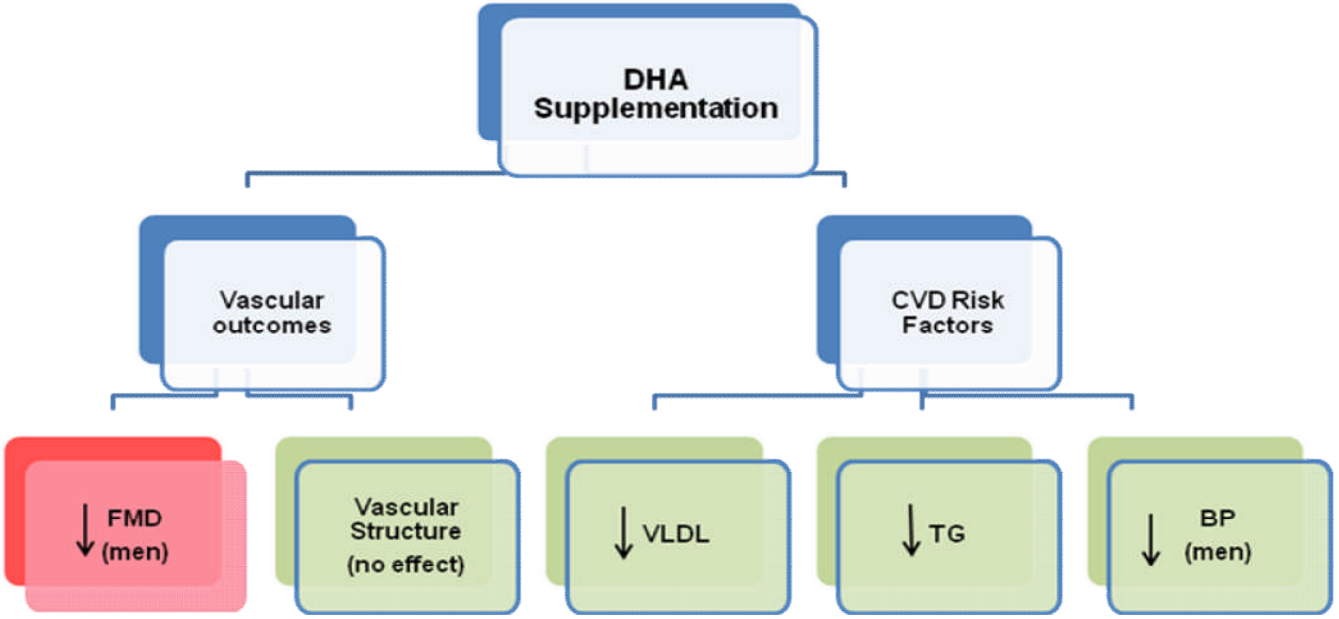
Figure 11-1 **Diagram summarising relationships of diet with vascular structure and function and CVD risk factors identified in the epidemiological study**



Abbreviations in diagram: DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; CVD RFs, cardiovascular disease risk factors; CCA-IMT, common carotid artery intima media thickness; BP, blood pressure; HR, heart rate; VLDL, very-low density lipoprotein cholesterol; TG, triglycerides; BA-Dist, brachial artery distensibility; FMD, flow mediated dilatation; WHR, waist to hip ratio; FBG, fasting blood glucose.

Green box denotes beneficial association, red box denotes adverse association.

Figure 11-2 **Diagram summarising effects of DHA supplementation on vascular structure and function and CVD risk factors in the RCT**



Abbreviations in diagram: DHA, docosahexaenoic acid; CVD RFs, cardiovascular disease risk factors; , FMD, flow mediated dilatation; VLDL, very-low density lipoprotein cholesterol; TG: triglycerides; BP, blood pressure.

Green box denotes beneficial effect, red box denotes adverse effect.

11.2 Discussion of Results

11.2.1 Dietary Patterns and Vascular Function

The finding that dietary patterns were not directly associated with brachial artery FMD was not unexpected. In **Chapter 2** an evidence review concluded that dietary components have both protective (e.g. flavonoids) and detrimental (e.g. saturated fat) effects on endothelial function. However, evidence from long-term trials was lacking. Whilst some observational studies have reported relationships of healthy dietary patterns with markers of EF these were all in older adults.[183, 287, 642, 643] No previous study of dietary patterns and FMD in healthy young people has been reported.

Other studies, although in older adults, support our findings and report no relationship between healthy diets and FMD. For example, Hodson and colleagues (2010) reported that the DASH diet, high in fruit, vegetables, wholegrains and low fat dairy foods, reduced BP but did not benefit FMD raising the possibility that benefits of a healthy diet for FMD operate via effects on other CVD risk factors.[241] Similarly Ambring (2004) found no benefits for FMD in a Swedish population following a Mediterranean diet.[233]

Markers of endothelial activation and inflammation can be used as surrogate measures of EF. Several studies have evaluated such markers to assess relationships between diet and plasma biomarkers of endothelial dysfunction.[287, 644, 645] The present study found that CRP (a marker of endothelial activation) increased as scores on the snack dietary pattern increased. In the Multi-Ethnic Study of Atherosclerosis (MESA), Nettleton and colleagues (2006) reported that a dietary pattern termed “fats and processed meats” characterised by high loadings for fats, oils, processed meats and fried potatoes, was positively associated with CRP (*P* for trend <0.001).[642] A second pattern, characterised by beans, tomatoes, refined cereals and high fat dairy products, was positively associated with soluble intracellular adhesion molecule (sICAM) -1 (*P* for trend 0.007). In contrast more healthy dietary patterns, characterised by high loadings for fruits, vegetables and

wholegrains were associated with lower concentrations of these and other markers.

Lopez-Garcia (2004) also found that a 'prudent' diet characterized by higher intakes of fruit, vegetables, legumes, fish, poultry and wholegrains was associated with lower concentrations of CRP (P = 0.02) and E-selectin (P = 0.001) (a marker of endothelial dysfunction).[183] Conversely, a 'western' dietary pattern characterised by higher intakes of red and processed meats, confectionary, fries and refined grains was positively associated with CRP (P < 0.02) and E-selectin (P < 0.001) as well as with soluble adhesion molecules sICAM-1 (P < 0.002) and sVCAM-1 (P = 0.02).

These observations support the hypothesis that dietary patterns are associated with EF indirectly and relationships are mediated through classical CVD risk factors.

11.2.2 Dietary Patterns and Vascular Structure

The finding that dietary patterns were associated with vascular structure was expected and has been shown in previous studies.[281, 626, 646] For example, The Insulin Resistance Atherosclerosis (ARIC) Study found that a dietary pattern characterised by higher intakes of less healthy foods (e.g. low fibre bread and cereal, red and processed meat, tomato foods and sweetened beverages) and lower intakes of more healthy foods (e.g. wine, rice and pasta and poultry) was associated with increased CCA-IMT in middle-aged adults (1 SD increase in dietary pattern score corresponding to 13 µm increase in CCA-IMT).[281] Our study is the first to identify this association in healthy young people. This is important because dietary patterns are established early in life and likely to track. Evidence to support this comes from the Young Finns Study where a traditional dietary pattern characterized by low intakes of fruits and vegetables in children aged 3-18 years was associated with increased IMT in adulthood.[646]

An unexpected finding in this present study was that higher arterial distensibility was associated with a snack dietary pattern in women. Possible explanations include ambiguity regarding the interpretation of this dietary pattern as healthy or not. For example, the snack pattern loaded high on convenient fish products and combination meals. Whilst some convenience foods may be high in nutrients that when taken in excess adversely affect CV health such as saturated fats others may not. In recent years the food industry has moved towards producing convenience foods that meet healthy eating guidelines. Examples include foods termed "Healthy Options" or labelled "Be Good to Your Self" widely available in most supermarkets. Therefore, convenient fish products that loaded high on the snack pattern may have contained oily fish. Similarly, convenience foods may have been healthy options as described above that were low in saturated fat and high in fruits and vegetables.

In secondary analyses the effect of a snack dietary pattern on CCA distensibility was confined to females raising the possibility of separate gender effects. An important consideration is the possibility that foods characterising the snack dietary pattern differ according to gender. For example in men convenience foods may be high in saturated fats whereas in women they may be a healthier option as discussed above.

Surprisingly, we found tomatoes were characteristic and loaded high on the meaty dietary pattern; a finding that appeared counter to current perceptions of unhealthy dietary components. However, as proposed by Liese and colleagues (2010), this could be explained by the inclusion of tomatoes in (usually high fat) convenience foods including pizza and hamburger relish.[281] In contrast the meat based dietary pattern in this study did not include sweetened beverages as in the less healthful diet described by Liese et al (2010).[281] Rather, these loaded high in the snack pattern.

11.2.3 Dietary patterns and classical CVD risk factors

In the present study, benefits for obesity, blood pressure, serum lipid concentrations and insulin resistance were suggested by reductions in these risk factors as scores on the healthy dietary pattern increased. In contrast, CVD risk factors were associated with higher scores on the meat and snack dietary patterns. A healthy dietary pattern was associated with lower BP and a less healthy dietary pattern with higher BP, a major determinant of arterial stiffness.[31] Therefore, diet may influence the development of vascular disease through effects on BP.

Dietary patterns have been shown to offer protection against high BP in previous studies; most notably, the DASH dietary pattern characterised by high intake of fruit, vegetables and low-fat dairy products, including wholegrains, poultry fish and nuts and low intake of red meat, sweets and sweetened beverages, has been associated with lower systolic and diastolic BP (5.5mm Hg and 3.0mm Hg respectively).[387] These associations are similar to those reported in the present study.

As in the present study, dietary patterns have been reported to benefit the lipid profile. For example, Hoffman and colleagues (2004) found HDL decreased across quintiles for a dietary pattern characterized by high consumption of meat, margarine and vegetable fats and oils. No trend was seen across quintiles of dietary pattern score for any other lipid fractions.[627] In contrast, reductions in TC, LDL, VLDL and TAG were seen across quintiles of health conscious dietary pattern score in this present study.

The present study also found associations of healthy dietary patterns with risk markers for T2DM. A higher score on a healthy dietary pattern was associated with lower fasting insulin concentration. This is supported by previous studies. For example, Fung and colleagues (2001) reported from the US Health Professionals Study that a “Prudent” dietary pattern, high in fruit, vegetables, wholegrains and poultry was associated with lower insulin concentrations.[644]

This epidemiological study found that diet was directly associated with measures of vascular structure such as CCA-IMT and arterial distensibility. A clear association of dietary patterns with vascular structure suggests diet may influence vascular health directly. Associations of dietary patterns with CVD risk factors including BP, HR, VLDL and TG were also found. This suggests diet may also influence vascular health through CVD risk factors. Path analyses would help elucidate these relationships. Sex differences were apparent and these warrant further investigation. Further follow up of this cohort into middle- and older-age, when clinical signs of CVD are likely to present, would clarify this relationship.

11.2.4 *n*-3 Fatty Acids and Vascular Function

Associations of *n*-3 fatty acids with FMD observed in the epidemiological dietary study were supported by findings of the RCT. In the epidemiological study, higher DHA status was associated with lower FMD in women and the RCT found that supplementation with DHA adversely affected FMD in men. These findings were not completely unexpected as a previous study reported lower FMD in women who were high fish consumers[270] and *n*-3 LC-PUFA supplementation has been shown to increase the concentration of soluble e-selectin, a marker of endothelial activation, in younger compared to older men.[234, 247] This suggests DHA has different effects on endothelial function according to gender.

11.2.5 *n*-3 Fatty Acids and Vascular Structure

There was no association of *n-3* fatty acid status on vascular structure in this study. This was unexpected as previous studies have reported associations of *n-3* LC-PUFA with CCA-IMT. However, most of these studies have been conducted in patients who already had established CAD[647-649] and findings from observational studies in healthy people are conflicting. For example, Djousse and colleagues (2003) found no associations of fish or *n-3* PUFA consumption with IMT in a large cohort (*n* = 1,575) of healthy Caucasians.[273] Conversely, Ebbesson and colleagues (2008) reported a protective effect of fish intake on IMT in Alaskan Eskimos, age 35 and older (*n* = 686).[274] These two studies differed in many ways.

Djousse (2003) studied individuals participating in a North American study (NHLBI Family Heart Study) who were chosen either at random or on the basis of a higher than expected risk of CAD. Ebbesson (2008) studied an indigenous population of Inupiat Eskimos who were largely village dwellers with a hunter-gatherer existence. Therefore, comparisons between these two studies are difficult to make as differences in lifestyle and health status may also have influenced vascular health. Furthermore, reliable conclusions cannot be drawn from the few studies currently published and further research is needed to define the relationship between *n-3* LC-PUFA consumption and vascular structure.

11.2.6 *n*-3 fatty acids and CVD risk factors

As with many previous studies benefits of *n*-3 LC-PUFA for classical CVD risk factors were suggested by associations of higher DHA status with lower levels of risk factors.

In the epidemiological study higher DHA status was associated with lower waist to hip ratio and FBG. TG and VLDL were lower in association with higher scores on the healthy dietary pattern and with higher EPA status. Evidence to support benefits of higher *n*-3 fatty acid status for CVD risk factors comes from the RCT where BP was lower in men and TG and VLDL were significantly lower in both sexes following supplementation with DHA. This is consistent with previous reports where DHA reduced TG concentration.[299, 386, 569] Although the reasons for sex differences are unclear our data our consistent with earlier studies and meta-analyses that showed low to moderate doses of DHA can reduce blood pressure in healthy people without affecting vascular function.[260, 386]

Heart rate was lower in association with higher EPA status and the strength was similar to that reported in a previous study.[274] However, for the first time in the present study this is seen in a young, healthy population. Improvements in heart rate have been reported previously with high doses of EPA and DHA

supplementation[650] and at lower doses, similar to that used in the RCT reported here.[651]

11.2.7 Mechanisms for Relationships of Diet with Endothelial Health

11.2.7.1 Dietary Patterns

In the epidemiological study dietary patterns were directly associated with vascular structure but not with FMD. This is surprising because endothelial dysfunction is the earliest preclinical sign of vascular disease and is widely reported to precede structural changes in the vascular endothelium.[11, 652] However, it is important to note that the study population were young healthy people in whom vascular changes may not yet be detectable. In the present study mean IMT was 0.5 mm which is at the lower end of the range in healthy young people.[653, 654] Furthermore, IMT shows little change in early life and increases in carotid IMT are reported to occur only once adulthood is reached. Therefore it is possible that a healthy diet helps maintain vascular integrity, which is reflected in a lower IMT, whilst a less healthy diet leads to very small changes that have no clinical impact in early adult life.

Dietary intervention studies have consistently shown that FMD is influenced in the short term by the fat content of a meal, probably as a consequence of increased triglyceride-rich lipoproteins and remnants that occurs following a high fat meal.[303] As discussed in **Chapter 3, Section 3.7.2.1**, these particles have a central role in atherogenesis and it is possible that repeated transient adverse effects on FMD lead to vascular changes in the long term. Hence, relationships between dietary patterns and FMD become apparent only in older adults.

A healthy dietary pattern was favourably associated with CVD risk factors. In particular, BP, HR, VLDL and TG were lower in association with higher scores on this pattern. These risk factors have previously been reported to adversely influence vascular function.[655] In this young healthy population, free from clinical CVD, these risk factors were within the normal range for most participants.

Therefore, it is likely that benefits of diet for endothelial health operate via CVD risk factors. In the absence of research investigating relationships between habitual diet and FMD in healthy young people conclusions are difficult to draw. However, it is likely that direct adverse associations of diet with FMD have yet to emerge.

11.2.7.1 *n*-3 Fatty Acids

In the RCT DHA was found to have a direct adverse effect on FMD in men. This was unexpected as improved FMD has been suggested as a key mechanism for the protective effect of *n*-3 LC-PUFA on the progression of atherosclerosis.[180, 235, 243, 256, 353, 430, 434, 571-575, 578] Mechanisms include direct vasodilatory effects reported in several short-term dietary intervention studies.[222, 226, 235]

11.2.8 Gender effects

The epidemiological study found that dietary patterns were associated with vascular structure in women but not in men. This may be due in part to a lower risk of CVD in premenopausal women compared with men **(Chapter 1, Section 1.5.5.1).** Benefits are largely proposed to operate through the female hormone oestrogen that has been shown to reduce oxidative stress and increase NO production.[656, 657 48] Premenopausal women also have higher concentrations of cardioprotective HDL than men. These features are likely to have favourable influences on the development of atherosclerosis. It is also widely reported that women are more likely to comply with dietary guidelines than men.[658, 659] In the present study the proportion of women in higher quintiles of the healthy dietary pattern score was greater than men. Therefore, small sample size with respect to men may partially explain the absence of an association of the healthy dietary pattern and vascular structure in men.

Our observation that higher DHA status in women was associated with a lower FMD was unexpected as reduced FMD is significantly related to male gender.[652] However, our finding is supported by Anderson, (2010) who reported a lower FMD

in women who consumed the highest quantity of fish.[270] A possible explanation is that high levels of methyl mercury found in fish may decrease the beneficial effects of *n-3* LC-PUFA. Evidence for this comes from the Kuopio heart study where a protective effect of *n-3* LC-PUFA on the risk of sudden cardiac death was attenuated by methyl mercury status.[288] It is possible therefore that in our study and that of Anderson (2010) high fish consumers also had high methyl mercury levels.

A further possible explanation is that hormonal fluctuations that occur during the menstrual cycle affected FMD in women in the present study.[660-662] Oral contraceptive use may also affect FMD.[623] In the present study we were not able to consider effects of the menstrual cycle or the use of oral contraceptives and it is possible that in this young population, where all females were premenopausal, that the menstrual phase was a confounding factor not accounted for.

The effect of DHA supplementation on men is also supported by earlier data showing detrimental effects of *n-3* LC-PUFA on endothelial activation in men.[234, 622]

The mechanism for different effects in men and women is unclear. Women have greater tissue DHA content than men[622] and a higher capacity to metabolise α-linolenic acid to DHA [623] However, research that aims to establish optimal fatty acid status in relation to vascular function is lacking.

The combined findings from this epidemiological dietary study and RCT suggest important gender differences in relationships between *n-3* fatty acids and vascular function which warrant further investigation. Our finding in the RCT of a 20% lower FMD in DHA supplemented men and in the epidemiological study that higher DHA status was associated with 27% lower FMD in women suggest that future trials of *n-3* LC-PUFA need to be powered to detect possible gender differences in cardiovascular outcomes.

11.2.9 Dietary Assessment of *n-3* LC-PUFA

In **Chapter 10** a newly developed FFQ for assessment of dietary *n-3* was found to be a valid tool. Moderate correlations were reported between each of the three dietary assessment methods and red cell membrane EPA concentration. An acceptable degree of correlation was also found for the FishFQ and 24-hour recall method with respect to DHA. Validity coefficients, obtained using the method of triads, for the FishFQ were moderate and compared favourably with previous studies. These results are discussed fully in **Chapter 10, Section 10-4.**

The FishFQ therefore provides a useful tool for assessment of n-3 LC-PUFA intake.

11.3 Implications for Public Health

11.3.1 Epidemiological Study

Our finding that a healthy dietary pattern is associated with lower risk of CVD, the number one cause of death in many developed countries, in young people has considerable public health implications. Dietary patterns are established early in life and track over time throughout childhood[646] and adulthood.[663] Therefore associations of diet with CVD are likely to persist with age. It is important that educational interventions to encourage the adoption of healthy dietary patterns start early and are incorporated into school curriculums from pre-school to adulthood. It is also important that consistent public health messages are provided to encourage healthy eating throughout the life course.

In this observational study a higher status for DHA was associated with lower FMD in women. This is consistent with the findings of a cross-sectional analysis in 3045 middle-aged adults where the highest quartile of non-fried fish consumption was associated with a smaller FMD in women, compared to the lowest quartile.[270] Collectively these findings raise concerns regarding advice for fish consumption. Current recommendations in the UK from SACN provide guidance at a population level. In view of concerns regarding toxicity these recommendations suggest

separate requirements for children and for women already pregnant or planning to conceive. Findings from the present study, supported by previous data also suggest separate guidelines for men and women (regardless of pregnancy status) may be appropriate.

Whilst conclusions regarding causality cannot be drawn from this observational dietary study, our findings are supported by data from the RCT conducted in parallel with the epidemiological dietary study.

11.3.2 Randomised Controlled Trial

To our knowledge, the present study includes the largest RCT to test the impact of DHA supplementation on endothelial function and CVD risk factors in a healthy young population. Our data support the hypothesis that higher DHA consumption has benefits for CVD risk factors such as blood pressure and VLDL and triglyceride concentrations. However, the effects of DHA are unlikely to include benefits for endothelial function at intakes achievable through dietary sources. Rather, our findings, which are supported by those in others, suggest caution should be applied when recommending supplements as adverse effects on FMD are possible.

Although the clinical implications of lower TG in young adults is uncertain, hypertriglyceridemia has been proposed as an independent risk factor for CVD[664, 665] and a target for interventions to reduce cardiovascular risk.[666] The cumulative effect of higher *n-3* DHA intake on triglyceride levels may therefore help in the primary prevention of CVD.

The clinical implications of sex differences are unclear. However, the joint findings in the present study of lower FMD for both women and men in association with higher DHA status raises questions with regard to an optimal intake for this fatty acid.

11.3.2 Dietary Assessment

Reliable assessment of LC-PUFA intake is essential in studies that aim to evaluate relationships between *n-3* intake and health outcomes. It is also important in clinical practice where knowledge of intake is essential in the assessment of dietary adequacy. This is particularly relevant to secondary prevention of CVD where *n-3* is most clearly shown to have benefits. The FishFQ is easy to administer and places minimal burden on study participants, clinical patients, researchers and clinicians. Therefore it is a useful addition in dietary *n-3* assessment that can be used in research and clinical practice.

11.4 Strengths and Limitations

11.4.1 Epidemiological Study

The strengths of the epidemiological study include the use of trained investigators most of whom were qualified in nutrition and/or dietetics and the use of biomarkers that enabled the most rigorous evaluation possible within the financial and time constraints of the study. Fatty acid status was validated by measurement of red cell membrane concentrations. This supported associations of higher fatty acid status with reduced concentrations of triglyceride and other atherogenic lipoproteins (e.g. VLDL).

There are also some limitations to consider however. Firstly, the sample may not have been representative of the wider population. A high proportion of participants were students and most were in higher social classes.

As discussed in **Chapter 4, Section 4.5.3,** dietary assessment is subject to error at several levels. There were additional sources of error in the present study. For example, measurement periods differed for dietary assessments based on FFQ and 24 hour recall. It was not possible to collect 24 hour dietary recalls during different seasons. Therefore foods contributing to usual *n*-3 LC-PUFA intake may have been missed. However, the use of biomarkers of fatty acid status with uncorrelated measurement error helped to minimise bias.

11.4.2 Randomised Controlled Trial

Randomised controlled trials provide the best form of evidence for the effectiveness of interventions. They enable researchers to control for a range of variables, minimize risk of bias and allow determination of causality. The main strengths of this RCT were study design, a large sample size, parallel group, double-blind, objective evidence of good compliance with dietary intervention, and objective, validated outcomes such as FMD. However, the RCT has several potential limitations. First, we studied a young population at low risk of CVD in whom dietary affects on CVD risk factors may not yet be apparent and where DHA supplementation may not improve further an already largely healthy endothelium. However, the study population was similar to our previous cross-sectional study which found strong associations between red cell DHA concentration and brachial artery FMD. [568] Moreover, the lack of any effect of DHA supplementation together with previous data from older adults at moderate CVD risk[253] suggest that DHA supplementation is unlikely to improve FMD in healthy adults irrespective of underlying cardiovascular risk.

Second, to aid compliance DHA supplements were given for only 4 months. Although longer periods of supplementation may be necessary for effects on some measures of vascular structure such as carotid IMT, [569] previously the effects of *n-3* LC-PUFA supplementation on endothelial function have been suggested in interventions ranging from two weeks [256] to 8 months.[243] Finally, because previous studies suggested stronger effects of DHA than EPA on vascular function,[243, 430, 568] we supplemented with only DHA and not also with EPA. However, the independent vascular effects of EPA and DHA are poorly understood, and require further investigation.[667]

11.4.3 Validation Study

Estimates of *n*-3 fatty acid intake using the new FishFQ were higher compared with EPIC and 24 hour recalls and could represent an overestimate. However, it is also possible that separation of foods previously grouped together and the inclusion of a

wider range of *n-3* rich foods enabled more accurate assessment. Furthermore, the 24 hour recall method provides cross-sectional data that may not reflect usual diet. The questionnaire demonstrated good reproducibility when re-administered four months later in the same population and correctly classified a high proportion of participants according to fatty acid status as measured by percentage total red cell membrane fatty acids.

11.5 Recommendations for future research

In view of potentially important gender differences it is recommended that further studies are powered to investigate separate effects of diet on CVD according to gender.

Longer term evaluation of relationships between diet and atherosclerosis is recommended. This could include follow up of the present cohort to investigate tracking of dietary patterns and risk factors.

Evaluation of diet throughout the life stages and its relationship with FMD is recommended. This includes analysis of the association of dietary patterns in childhood with FMD later in life.

n-3 supplementation studies at doses obtainable at usual dietary intakes are needed to further inform the role of *n-3* fatty acids in primary prevention of atherosclerosis. These should be adequately powered to accommodated analyses by gender and should include supplementation with DHA and EPA.

Further studies of dietary *n-3* consumption and effects on vascular health are needed with particular attention to interactions with methyl mercury.

Further validation of dietary assessment methods is essential in all studies evaluating diet and relationships with CVD risk factors including vascular structure and function.

11.6 Conclusions

Diet has important effects on vascular health and CVD risk factors. In particular healthy dietary patterns may protect the vascular endothelium from structural changes associated with atherosclerosis. The mechanism is likely mediated through reductions in CVD risk factors including BP and atherogenic lipoproteins.

Dietary *n-3* fatty acids may also protect vascular health but direct associations and effects of supplementation are not apparent in young healthy adults and it is likely that their effect is mediated through conventional CVD risk factors. In contrast high intakes or supplementation may be detrimental for vascular function. Although it should be considered that supplementation may reduce overall CVD risk by achieving a reduction in other CVD risk factors. Most importantly, a reduction in TG seen in association with higher *n-3* status and as a result of DHA supplementation, at a dose within the normal dietary range, supports the role of *n-3* LC-PUFA in primary prevention of CVD.

Acknowledgements

I would like to gratefully acknowledge the help and advice I received from the following people:

Atul Singhal
Alan Lucas
Margaret Lawson
Ailsa Welch
Claire Robertson
Kate Northstone
Tim Cole
Sarah Collins
Toni Birbara
Ian Merryweather
Nigel Fuller
Maria Kokorelli
Laura Schwadtke
Maria Kolotorou
Kavita Shah
Jane Williams
Teagan Darch
Stuart Sabatini
John Deanfield
Ann Donald
Clare Storry
Ravneet Phalora
Elizabeth Ellins
Rebecca Mant
Karen Hubbard
Pauline Styles

I would also like to thank Kellogg's PLC, Manchester, UK for providing a charitable donation towards this research and Martek Biosciences Corporation (Maryland, US, now DSM Nutritional Lipids) for providing the DHA supplements used in the RCT.

I would especially like to thank all the people who gave up their time to willing participate in the RCT including my own children, Kerry, Joe and Harrison, their partners and friends.

Thanks to all my family and friends for their help and support.

Finally many thanks to Jeff Coyte for all your help and support in achieving this research and thesis.

Information of work in this dissertation

Conception and Design

The original idea for the RCT was conceived by Professors Atul Singhal and Alan Lucas. The dietary assessment arm of the RCT and the epidemiological dietary study was conceived and designed by me.

Author's role

My roles in this project were trial co-ordinator for the RCT and Principal Investigator of the epidemiological study. This involved protocol development and preparation and submission for ethical approval in collaboration with my primary supervisor Professor Atul Singhal. I was responsible for management and day to day running of the RCT and epidemiological study including aspects detailed below.

Staff Management and Training

I managed and supervised all CNRC staff working on the project.

Data Collection

I undertook and/or supervised the following aspects of data collection:

Obtained informed consent for study participation and provision of blood samples.
Collected biological samples including DNA, blood and saliva.
Completed questionnaires including medical history, lifestyle and dietary.
Conducted anthropometric measurements.
Conducted or assisted in vascular ultrasound measurements.
Conducted compliance monitoring.
Managed reporting of adverse events.
Participated in collection of 24-hour recalls.

Data Analysis

Design of data collection forms.
Collated data from collaborating departments and laboratories.
Assembled master database.
Cleaned and checked data.
Analysed all data.

Thesis construction

The final thesis was designed and written by me. Help in proof reading was provided by Becky Mant and Karen Hubbard.
Help with formatting was provided by Pauline Styles.

Appendix 1-1 Search strategy for MEDLINE using OVID interface

Dates January 1992 to June 2012

Limits English language and humans

1. Exp atherosclerosis
2. Cardiovascular risk
3. Coronary artery disease
4. exp vascular endothelium/
5. exp vascular endothelial function/
6. exp vascular endothelial dysfunction/
7. endothelial dependent relaxation/
8. exp endothelium dependent vasodilatation/
9. exp endothelium independent relaxation /
10. exp endothelium independent vasodilatation/
11. exp flow mediated vasodilatation/
12. exp FMD/
13. exp/EF
14. exp.vascular structure
15. exp.pulse wave velocity
16. exp Sodium nitroprusside/
17. exp forearm blood flow/
18. exp acetylcholine/
19. exp vessel wall volume/
20. exp IMT carotid/
21. icam vcam
22. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21
23. *diet/
24. *nutrition/
25. *food/
26. *meal/
27. dietary supplements/
28. 23 or 24 or 25 or 26 or 27
29. 22 and 28
30. exp intervention studies/
31. exp randomised controlled trials/
32. exp cohort studies/
33. exp comparative studies/
34. exp prospective studies
35. 30 or 31 or 32 or 33 or 34
36. 22 and 35
37. 28 and 36
38. 29 and 35

Appendix 1-2 Tables of Studies Included in Systematic Review of Diet and Endothelial Health

Table 2-1 Studies investigating effects of short-term (single meal or supplement) dietary interventions on endothelial structure and/or function in healthy individuals

Author, Setting, (year)	Study Design	Dietary Intervention	Outcome Measures	Results
Bondonno, Australia, (2011).	R, CO trial in 30 healthy men and women	Effects of four energy matched treatments (control, apple, spinach and apple + spinach) on vascular function compared.	Brachial artery EDV assessed by FMD.	Compared to control all treatments resulted in higher FMD. Increase for apple: (1.1%, 95% CI: 0.7 – 1.5, p<0.05)
Fahs, US, (2010).	R, PC study of 20 healthy men and women (10 in each group).	HF meal plus 1g EPA and DHA or placebo.	Brachial artery EDV assessed by FMD and arterial stiffness.	FMD significantly impaired following HF meal with placebo (p=0.03). No effect on arterial stiffness.
Vauzour, UK, (2010).	R, CO, PC study of 15 healthy adult men and women.	Champagne wine compared with control matched for alcohol, CHO and fruit derived content given as single ingestions.	Brachial artery EDV and EIDV measured using iontophoresis.	Independent vascular reactivity increased more at 4 (p=0.030) and 8 (p=0.045) hours following champagne consumption compared with control.
Crecelius. USA, (2010).	PC study of 14 healthy older men.	Acute administration of various doses of ascorbic acid during 10 minute exercise period.	FBF measured using Doppler ultrasound.	Ascorbic acid administration increased FBF minimally during exercise via an increase in NO availability (p=NS).
Tousoulis, Greece, (2010).	R, parallel study. 37 healthy participants, mean age 28 years (26 men 11 women).	50 mls maize, cod liver, olive or soya oil or water given as single test meal.	FMD in the right forearm using gauge-strain plethysmography and serum concentrations of VCAM-1 measured pre and post prandially.	Cod liver oil and soya oil improved hyperaemia after 1 hour compared with control (p<0.05). Olive oil reduced FMD. No effect on VCAM-1.

Author, Setting, (year)	Study Design	Dietary Intervention	Outcome Measures	Results
Jackson, UK, (2009).	24 healthy men (12 younger (<50 years) and 12 older (>50 years).	Single meal test meal providing 5.4g EPA and DHA.	Peripheral microvascular EDV and IEDV after Ach and SNP infusion assessed before and after meal.	Fish meal improved EF in younger men 4 hours after Ach and SNP (p<0.04).
Kelly, Singapore, (2008).	R, CO, PC study in 26 healthy volunteers (18 men and 8 women).	Beverage containing 2g vitamin C compared with lemon juice placebo.	PWF following GTN.	No effect of vitamin C on any measures of vascular structure or function.
Alexopoulos,. Greece, (2008).	Controlled study of 14 healthy adults, mean age 30 years.	Green tea beverage.	Brachial artery EDV assessed by FMD.	FMD increased 30 minutes after tea consumption (3.69% [p<0.02]).
Jochman, Germany (2008).	R, CO, PC study of 21 healthy men and women.	Black and green tea beverages.	Brachial artery FMD	FMD increased after both green and black tea. Difference in FMD 5.0 (95% CI 3.0, 7.0, p<0.001) and 4.4 (95% CI 2.3, 6.5; p<0.001) respectively.
Armah,. UK, (2008).	R, controlled study of 25 healthy men; mean age 46 years.	Single test meal comparing fish oil (5.4g EPA/DHA) with placebo oil.	EDV and IEDV assessed for measurement of SNP induced RH.	SNP induced RH increased with *n*-3-PUFA compared with standard diet (p=0.024).
Berry, UK, (2008).	Controlled study of 17 healthy men; mean age 18-40 years.	High stearic acid compared with high oleic acid meals.	Brachial artery FMD.	FMD decreased with oleic compared with stearic acid -3.0% (95% CI -4.4, -1.6, p<0.001).
Mariotti,. France, (2007).	R, CO, PC study of 9 healthy young men – age not reported.	50g meal supplemented either with (intervention) or without (control) 3g L-arginine added.	Brachial artery FMD, CCA distensibility and PWV measured with vascular ultrasound.	No alteration in EF related to timing or content of test meals.
Shimabukuro, Japan, (2007).	Controlled study of 12 healthy adults; mean age 36 years, (6 men and 6 women).	High CHO, compared with HF and standard meal.	FBF measured during RH before and after meals.	Peak FBF decreased after high fat meal (p<0.01). No difference after high CHO or standard meal.

Author, Setting, (year)	Study Design	Dietary Intervention	Outcome Measures	Results
Nicholls, Australia, (2006).	Controlled study of 14 healthy adults; aged 18-40 years; (sex not reported).	High SFA compared with high PUFA (fatty acids not defined) meal.	FBF and EDV assessed by brachial artery FMD.	No difference.
Padilla, USA, (2006).	Controlled study of 8 healthy men and women; mean age 26 years. (5 men, 3 women).	Effect of aerobic exercise on LF, and HFM induced decreases in FMD	Brachial artery EDV assessed by FMD 4 hours post intervention.	FMD increased after HFM following exercise (5.61%, p=0.005). No difference following LF and HFM only.
Tushuizen, Netherlands, (2006).	Controlled study of seventeen healthy men, mean age 25.4 years.	Standardized HFM (50 g fat, 55 g CHO and 30 g protein) given twice (lunch and breakfast).	Brachial artery EDV assessed by FMD pre and post meal.	FMD significantly impaired following 2nd HFM (6.9% vs.3.7%, p<0.05).
Westphal, Germany, (2006).	Controlled study of 16 healthy young adults; aged 19-23 years, (8 men, 8 women).	Effects of HFM, HFM + soya protein, HFM+ casein compared.	Brachial artery EDV assessed by FMD.	FMD decreased after HFM (maximum decrease 58%). Addition of protein to meals attenuated effect (p<0.01).
Karatzi, Greece, (2005).	R, CO, DB, PC study of 16 healthy subjects (8 men and 8 women) mean age 29 years.	Coffee containing 80 mg caffeine compared with decaffeinated coffee control.	Arterial stiffness assessed using PWA to give augmentation index (AI).	AI index increased more following caffeine consumption (indicating unfavourable effects) compared with placebo (p= 0.001).
Tsai, Taiwan, (2004).	Controlled study of 16 healthy males; mean age 30 years.	HFM.	Brachial artery EDV assessed by FMD.	FMD decreased after high fat meal (data not presented).
Vlachopoulos, Australia, (2003).	R, CO, PC study of 20 healthy subjects.	250 mg caffeine compared to placebo.	Arterial stiffness assessed using PWA to give augmentation index (AI).	PWV increased by 0.51 m/s and AI by 6.8% for intervention compared with control group (p<0.001 for both variables),indicating an unfavourable effect of caffeine.

Author, Setting, (year)	Study Design	Dietary Intervention	Outcome Measures	Results
Waring, UK, (2003).	R, DB, PC study of 20 healthy adults.	300 mg caffeine compared with placebo.	Arterial stiffness assessed using pulse wave analysis to give augmentation index (AI).	Acute caffeine intake increased AI significantly compared with placebo (7 ± 2%, $p<0.05$).
Steer, Sweden, (2003).	Controlled study of 26 healthy adults; aged 20-30 years.	High, medium and increased fat meals compared.	FBF determined with venous occlusion plethysmography. EDV and EIDV assesed.	EDV increased after LF ($p<0.01$). FBF and EDV decreased after HFM ($p<0.01$ and $p<0.05$ respectively.
Bae, South Korea, (2003).	Controlled study of 10 healthy males; mean age 26 years.	HFM.	Brachial artery FMD.	No effect on FMD 3-4 hours post meal.
Fisher, US, (2003).	Controlled study of 27 healthy men and women (11 males and 16 women), mean age 44 years.	920 mls cocoa consumed in four oral doses over a 5-day period.	PWA assessed 90 minutes following cocoa consumption.	After 5 days of cocoa consumption PWA increased ($p= 0.009$).
Nappo, Italy, (2002).	Controlled study of 20 healthy adults, mean age 44 years (10 in each group).	Two single meals given at 1 week intervals comparing high fat with high CHO diet.	Inflammatory markers and VAM.	High fat meal increased TNFα, IL6, ICAM-1 and VCAM-1.
De Roos, Netherlands, (2002).	Controlled study of 21 healthy young males; aged > 35 years,	High SFA compared with high-trans-fatty acid meal.	Brachial artery EDV assessed by FMD.	No difference between meals.
Hashimoto, Japan, 2001.	Controlled study of 11 healthy men, mean age 34 years.	Participants randomised to 1 of four beverages: Japanese vodka, red wine with alcohol, red wine without alcohol or water.	Brachial artery FMD measured 30 and 120 minutes following ingestion.	Significant increases in RH 120 minutes after ingestion of red wine without (increase 0.1% [$p<0.01$]) and with alcohol (increase 0.6%, $p<0.01$).
Marchesi, Italy, (2000).	Controlled study of ten young healthy men, mean age 23 years.	Post-prandial (2, 4, 6 and 8 hours) effects of HFM on FMD.	Brachial artery FMD measured before and after meals.	FMD decreased from 14.5 ± 6.6% fasting to 3.5 ± 1.5% and 4.0 ± 2.2% at 2 and 4 hours ($p<0.01$).

Author, Setting, (year)	Study Design	Dietary Intervention	Outcome Measures	Results
Vogel, US, (2000).	R, CO, PC study in 10 healthy participants aged 28-56 years.	5 meals with 50g fat from various sources: olive oil, rapeseed oil, canned red salmon.	Brachial artery FMD measured before and 3 hours after meals.	FMD reduced after olive oil meal, 31% (14.3 ± 4.2% to 9.9 ± 4.5%, p=0.008).
Cuevas, Chile, (2000).	Controlled study of 11 healthy men; aged 20-28 years.	HFM +/- red wine compared with standard diet +/- red wine.	Brachial artery EDV assessed by FMD.	FMD decreased with HFM minus red wine compared with control diet (p=0.014).
Raitakari, Australia, (2000).	Controlled study of 7 healthy men and women; aged 18-45 years, (4 men, 3 women).	SFA rich compared with MUFA meal.	FBF and brachial artery EDV assessed by FMD.	FBF increased after both meals (p<0.001 for all meals). No difference in FMD according to meals.
Djousse, USA, (1999).	Controlled study of 13 healthy men and women; mean age 32 years.	HFM with wine compared with HFM and control beverage.	Brachial artery EDV assessed by FMD.	No difference between wine and control beverage.
Ong, UK, (1999).	R, DB, CO study in 10 healthy males; aged 30.	HF/L-CHO compared with LF/H-CHO meal.	Brachial artery EDV assessed by FMD.	FMD decreased after HF/L-CHO compared with LF/H-CHO meal, (1.2% vs. 4.3% p=0.02)
Williams, New Zealand, (1999).	Controlled study of 10 healthy males; aged 34-52 years.	LFM, HFM and deep fried meals compared.	Brachial artery EDV assessed by FMD.	FMD decreased after deep fried meal (5.9 ± 2.3% vs. 0.8 ± 2.2 %, p=0.0003). No difference with high fat or low fat meal.
Vogel, USA, (1997).	Controlled study of 10 healthy adults; age and sex not reported.	HF compared with LF meal.	Brachial artery EDV assessed by FMD.	FMD decreased after high fat (difference from baseline to 4 hours compared with low fat meal -11%, p<0.05).

Abbreviations in table: R, randomised; CO, crossover; DB, double-blind; PC, placebo controlled; LF, low fat, HF, high fat; CHO, carbohydrate; L-CHO, low carbohydrate, H-CHO, high carbohydrate; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EDV, endothelium-dependent vasodilatation; EIDV, endothelium-independent vasodilatation; FMD, flow mediated dilatation; FBF, forearm blood flow; PWA, pulse wave amplitude; PWV, pulse wave velocity; AI, augmentation index; RH-PAT, reactive hyperaemia peripheral arterial tonometry; VAM, vascular adhesion molecule; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM, soluble intracellular adhesion molecule-1; CCA, common carotid artery; IMT, intima media thickness; Acth, Acetylcholine; SNP, sodium nitroprusside; NO, nitric oxide; GTN, glyceryl trinitrate; TNF-α, tumour necrosis factor alpha; IL-6, interleukin 6.

Table 2-2 Studies investigating effects of longer-term dietary interventions (two weeks to two years) on endothelial function in healthy individuals

Author, Setting (year)	Study Design	Dietary Intervention and Assessment	Outcome Measures	Results
Thies, UK, (2012).	RCT in 225 healthy volunteers (94 men and 131 women).	Three dietary groups: low (control) or high tomato-based and control plus 10 g/d lycopene supplement for 12 weeks.	Arterial stiffness.	No effect on arterial stiffness.
Sanders, UK, (2011).	RCT in 312 healthy participants, aged 45-70 years.	EPA/DHA supplements (0.4, 0.9 and 1.8 g/d) compared with olive oil placebo for 1 year. **3-day FR.**	Brachial artery EDV assessed by FMD.	No difference in FMD between intervention and control groups.
Marin, Spain, (2011).	RCT in 20 healthy elderly participants (10 men, 10 women).	High SFA diet, L, H-CHO diet and MedDt compared after 4 weeks intervention. **3-day WFR and FFQ.**	EMP and EPC measured to assess endothelial activation.	MedDt led to lower EMP and higher EPC concentrations compared with other diets (p<0.001).
Morand, France, (2011).	RCT in 24 healthy men aged 50-65 years.	Hesperidin (flavonoid) compared with orange juice and placebo given for 3 four-week periods. **Not reported.**	MV RH assessed following each treatment period. PP effects assessed at start of each treatment period.	Hesperidin and orange juice both improved post-PP RH compared with placebo (p< 0.05).

Author, Setting (year)	Study Design	Dietary Intervention and Assessment	Outcome Measures	Results
Kim, Korea, (2011).	RCT in 126 healthy men aged 22–57 years.	Intervention group randomised to receive 15 mg lycopene/day for 8 weeks (n =37) compared with placebo controls (n = 41). **Not reported.**	EDV assessed by RH-PAT. Oxidative stress assessed by measurement of sICAM-1 and sVCAM-1.	RH-PAT increased: 23% (1.49 ± 0.09 vs. 1.79 ± 0.12 p = 0.03). VAM decreased in intervention vs. control group (p <0.05).
Din, UK, (2011).	RCT in 30 healthy men mean age 23 years.	Intervention group allocated to 15 g/day walnuts compared with no walnuts for 4 weeks.	Arterial stiffness assessed using PWA to determine AI.	No difference in AI according to randomised group.
Hodson, UK, (2009).	RCT in 27 healthy men and women aged 25–60 years.	Intervention group randomised to DASH diet or control group for 30 days. **Not reported.**	Brachial artery EDV assessed by FMD.	No effect on FMD.
Van Mierlo, Netherlands, (2010).	RCT in 35 healthy males aged 18-45 years.	Three 2-week intervention periods with 1-week washout periods comparing wine grape with grape only solids. **Not reported.**	Brachial artery EDV assessed by FMD.	FMD increased with wine grape solids: (difference -0.4%: 95% CI 1.8, 0.9; p = 0.77) compared with placebo.
Shai, Israel, 2010.	RCT in 140 subjects participating in the DIRECT-Carotid trial (123 men, 17 women), mean age 50 years.	Participants allocated either to LF MedDt or low CHO for 2 years. **Semi-quantitative FFQ.**	CCA-IMT and VWV.	Reduction in VWV reported with both diets: (mean difference -58.1 mm, 95% CI: -81.10 - 81.0. (p <0.001).
Tarcin, Turkey, (2009).	Case-control study comparing 23 healthy vitamin D deficient men and women aged 23 years (65% male), mean age 23 years with matched controls.	300,000 IU vitamin D given intramuscularly monthly for 3 months. **No dietary assessment reported.**	Brachial artery EDV assessed by FMD.	Significant increase in FMD for treatment group mean increase 10.4, SD 3.3%, (p<0.001).

Author, Setting (year)	Study Design	Dietary Intervention and Assessment	Outcome Measures	Results
Pot, Netherlands, (2009).	RCT in 77 healthy participants, mean age 59 years.	1.3 g/day EPA+DHA or placebo (sunflower oil) for 12 weeks. **Dietary assessment not applicable.**	Fasting serum concentrations of sVCAM, sICAM.	No effect of fatty acids on VAMs.
Grassi, Italy, (2009).	RCT in 19 healthy men aged 19–70 years.	Black tea given in doses ranging from 100-800 mg/day for 5 weeks. **Dietary assessment not applicable.**	Brachial artery EDV assessed by FMD.	FMD increased after all doses and in a dose dependant manner (P = 0.0001).
Sari, Turkey, (2009).	Controlled CO study of 32 healthy adult men aged 21-24 years.	MedDiet for 4 weeks + 4 weeks enriched with pistachio. **Nut consumption and use of supplements assessed.**	Brachial artery EDV assessed by FMD.	EDV increased after pistachio-enriched diet compared with MedDt (P = 0.002).
Miller, USA, (2009).	CO study of 18 healthy men and women; mean age 31 years, (9 men, 9 women).	HF/L-CHO (Atkins), Med Dt (South Beach) and LF (Ornish) diets compared using 3, 4-week dietary interventions with 4-week wash out. **3-day food record per intervention period.**	Brachial artery EDV assessed by FMD.	Inverse correlation of FMD with SFA (r = 0.33; P = 0.016)
Rallidis, Greece, (2009).	RCT in 82 healthy men and women (age not reported).	MedDt +/- dietary advice and adherence monitoring for 2 months. **3-day diet records and 24 hour dietary recall.**	Brachial artery EDV assessed by FMD.	FMD increased in intervention compared with control group: (2.05%; 95% CI: 0.97 – 3.13%).

Author, Setting (year)	Study Design	Dietary Intervention and Assessment	Outcome Measures	Results
Hall, UK, (2008).	Controlled study of 110 healthy adults; aged 30-70 years (sex not reported).	High-SFA, compared with high-MUFA compared with LF/H-CHO. Duration: 6 months. **Not reported.**	Brachial artery FMD, DVP and PWV.	No differences in FMD or PWV. DVP decreased with LF/high CHO compared with high SFA and MUFA.
Theobald, UK (2007).	RCT in 38 healthy women aged 40-65 years.	DHA (0.7 g/d in 1.5 g/d capsule) compared with olive oil placebo (1.5 g/d). Given for 3 months. **3-day dietary record.**	EIDV assessed following GTN administration. Arterial stiffness assessed from digital pulse volume.	No effects of DHA on measures of vascular structure or function.
Rueda-Clausen, Colombia, (2007).	RCT in 10 healthy men; aged 18-23 years.	HF meals: palm, olive, sunflower oil +/- deep frying. Duration: 9 weeks. **Weekly 24 hour dietary recalls.**	Brachial artery EDV assessed by FMD.	FMD decreased after each meal (32.1%; 95% CI: 28.0 – 36.2), no further effect of deep frying.
Shah, US, (2007).	RCT in 26 healthy men and women; mean age 31 years, (17 men, 9 women).	Supplemental n-3-PUFA (fish oil) compared with corn oil. Duration: 14 days. **Not reported.**	Brachial artery EDV and EIDV assessed by FMD.	FMD and EIDV increased with FO compared with corn oil (20.4% ± 13.2% vs. 9.9% ± 5.4%; *P* = 0.036).
Cazzola, Italy, (2007).	P, DB study in 93 healthy young men, mean age 26 years.	1.35 or 2.7 or 4.05 g EPA/day, compared with placebo (corn oil) for 12 weeks. **Not reported.**	Fasting serum concentrations sVCAM-1, sICAM-1, E-selectin.	4.05 g/d EPA/DHA increased sE-selectin in younger men.
Egert, Germany, (2007).	P, SB study in 48 healthy participants aged 18-45 years, (13 men, 35 women).	Dietary study comparing 6 g ALA, 3 g EPA or DHA/day for 3 weeks. **3-day dietary record.**	Fasting serum concentrations sVCAM-1, sICAM-1, sE-selectin.	No effect on outcomes.

Author, Setting (year)	Study Design	Dietary Intervention and Assessment	Outcome Measures	Results
Walser, US, (2006).	P, SB study study in 13 healthy participants.	EPA (3 g/d + DHA (2 g/d), compared with placebo (safflower oil) for 6 weeks. **Not reported.**	Exercise-induced increases in brachial artery diameter and blood flow.	Fish oil enhanced contraction-induced increases in brachial artery diameter, blood flow and conductance.
Keogh, Australia, (2005).	R, CO trial of 40 healthy adults; aged 40–75 years, (19 men, 21 women).	Low fat/high CHO vs high fat (high SFA), high MUFA , compared with high PUFA. Duration: 3 x 4 weeks. **3-day WFR.**	Brachial artery EDV assessed by FMD.	FMD decreased on SFA compared with all other diets.
Turner, Denmark, (2004).	RCT in 75 healthy volunteers aged 40-60 years.	Assigned to dried garlic powder containing 10.8 mg alliin (~ 3 cloves) compared with placebo. **Not reported.**	Arterial stiffness assessed with PWV measurement.	No significant decreases in outcomes between garlic and placebo groups.
Sharman, Australia, (2004).	R, CO study of 16 healthy men (mean age 58 years).	Supplement of 600 mg/d alpha-lipoic acid given on two separate occasions compared with placebo. **Not reported.**	Arterial stiffness assessed through pulse form analysis (PWV and AI).	No significant changes on any measures of arterial stiffness.
Eschen, Denmark, (2004).	P, DB study in 60 healthy participants, mean age 38 years.	5.9 g or 1.7 g/d EPA+DHA, compared with placebo (olive oil) given for 12 weeks. **Not reported.**	Fasting serum concentrations sVCAM-1, sICAM-1, sE-selectin.	5.9 g/d EPA+DHA decreased sP-selectin in men.
Ambring, Sweden, (2004).	R, CO study of 22 healthy men and women (12 men, 10 women) aged 30-51 years.	MedDt given for 4 weeks compared with usual Swedish diet. **Not reported.**	EIDV assessed with FBF.	No difference between dietary groups.

Author, Setting (year)	Study Design	Dietary Intervention and Assessment	Outcome Measures	Results
Rasool, Malaysia, (2003).	RCT in 20 postmenopausal women, mean age 54.6 years.	Supplement of tocopherol (400 IU) daily compared with placebo at intervals of 5 weeks. **Not reported.**	Arterial stiffness assessed through measurement of PWV.	No effect on PWV.
Khan, Scotland, (2003).	R, CO trial in 173 healthy men and women; aged 40-65 years (117 men, 56 women).	Supplemental *n*-3-PUFA, vs MUFA/PUFA, vs EPO, vs Soya bean oil. LF/H-CHO tuna/EPO mix corn oil vs control. Duration: 8 months. **FFQ and 24 hour dietary recall**.	EDV and EIDV measured in forearm skin with iontophoresis.	EDV increased after tuna FO supplementation ($P = 0.02$).
Lind, Sweden, (2002).	Controlled, CO study of 19 healthy men and women; aged 30-65, (13 men, 6 women).	High SFA, compared with high *n*-3-PUFA. Duration: 4 week cross over. **Not reported.**	FBF.	FBF increased after high SFA diet compared with high *n*-3-PUFA ($P < 0.05$).
Sejda, Czech Republic, (2002).	Controlled CO study of 11 healthy men and women; mean age 24 years, (6 men, 5 women).	LF compared with HF diet. Duration: 4 weeks **3-day FR – 5 points.**	Brachial artery EDV assessed by FMD.	No difference.
De Roos, Netherlands, (2002).	R, study of 29 healthy young men and women; mean age 30 years (10 men, 19 women).	High SFA, compared with trans-MUFA. Duration: 4 weeks. Duplicate meals.	Brachial artery EDV assessed by FMD.	FMD decreased more after trans-MUFA compared with H-SFA (difference 0.7%; 95% CI: -0.6 – 1.9)

Author, Setting (year)	Study Design	Dietary Intervention and Assessment	Outcome Measures	Results
Miles, UK, (2001).	Randomized controlled trial in 28 healthy participants grouped according to age: <40 and >55 years.	1.2g/d fish oil: EPA+DHA or placebo oil (palm and soya oil mix) given for 12 weeks. **Not reported.**	Fasting plasma sICAM-1, sVCAM-1, sE-selectin.	Fish oil increased sE-selectin in young males (median increase 38%; *P* = 0.043) and decreased in older males (median decrease 11%; *P* = 0.075). sVCAM decreased in older males (median decrease 20%; *P* =0.043).
Thies, UK, (2001).	P, DB study in 46 healthy participants, mean age 63 years.	2 g ALA or 0.7g GLA, AA or DHA/day, or 1 g/day. EPA/DHA or placebo (palm and sunflower oil mix) given for 12 weeks. **Not reported.**	Fasting plasma sICAM-1, sVCAM-1, sE-selectin.	ALA and EPA/DHA decreased sE-selectin and sVCAM-1.
Teede, US, (2001).	R, DB trial in 213 healthy subjects (108 men, 105 women) aged 50-75 years.	40 g soy protein (118 mg isoflavones) compared with casein placebo for 3 months. **Not reported.**	PWV and brachial artery FMD.	Compared with casein, PWV improved in soy group (*P* < 0.01). FMD declined in males in soy group (*P* < 0.02).
Chin, Australia, (1993).	P, SB, PC trial in 29 healthy males, age 18-32 years.	5, 10 or 20 g MaxEPA (0.18 g EPA + 1.2 g DHA/day, vs placebo. Duration: 4 weeks. **Not reported.**	Peripheral microvascular function assessed using iontophoresis and venous occlusion plethysmography.	Fish oil suppressed vasoconstriction in a dose-response manner: (20 g reduced by 72%, 10 g by 67%, 5 g by 33%)

Abbreviations in table: RCT, randomised controlled trial; R, randomised; P, parallel; CO, crossover; DB, double-blind; SB, single blind; PC, placebo controlled; LF, low fat; HF, high fat; MedDt, Mediterranean diet; DASH, dietary approaches to stop hypertension; CHO, carbohydrate; FA, fatty acids; SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids, EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALA, alpha linolenic acid; FFQ, food frequency questionnaire; FR, food record; WFR, weighed food record; EDV, endothelium-dependent vasodilatation; EIDV, endothelium-independent vasodilatation; FMD, flow mediated dilatation; FBF, forearm blood flow; PWA, pulse wave amplitude; PWV, pulse wave velocity; AI, augmentation index; DVP, digital volume pulse analysis; MV, microvascular; RH-PAT, reactive hyperaemia peripheral arterial tonometry; VAM, vascular adhesion molecule; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM, soluble intracellular adhesion molecule-1; CCA, common carotid artery; IMT, intima media thickness; VWV, vessel wall volume; GTN, glyceryl trinitrate.

Table 2-3 Observational studies investigating relationships between diet and endothelial structure and function in healthy people

Authors, Setting (year)	Subjects	Methods	Dietary Assessment	Results
van de Laar, Netherlands, (2012a).	Longitudinal cohort study of 373 healthy individuals age 13-36 years.	Dietary intake assessed during adolescence and adulthood. CCA stiffness (distensibility coefficient) measured at age 36 years.	Semi-quantitative FFQ.	Arterial distensibility related to lower fibre consumption (-1.9; 95% CI: -3.1 - 0.7).
van de Laar, Netherlands, (2012b).	Longitudinal cohort study of 373 healthy individuals age 13-36 years.	Dietary intake as above used to categorise participants in tertiles according to adherence to MedDt (aMED).	Semi-quantitative FFQ	Low aMED associated with lower arterial distensibility (-0.32, 95% CI: -0.60 - -0.06).
Crichton Australia, (2012).	Cross sectional study of 587 participants in the Maine-Syracuse Longitudinal Study.	Relationship between dairy food intake and PWV assessed.	Intake of dairy food assessed using the Nutrition and Health Questionnaire.	Linear decrease in carotid-femoral PWV seen with increased dairy consumption indicating beneficial effect.
Jablonski, US, (2010).	Cross-sectional study of 75 middle aged/older men and women.	Plasma 25-hydroxyvitamin D and 1,25 dihydroxy vitamin D used as a biomarkers of vitamin D status.	3-day food records.	Plasma 25-hydroxyvitamin D was positively associated with brachial artery FMD ($P < 0.01$). No significant association found for 1,25 dihydroxy vitamin D.
AlMheid, US, (2011).	Cross-sectional study in 554 healthy men and women.	Plasma 25-hydroxyvitamin D was used as a biomarker of vitamin D status.	None reported.	Plasma 25-hydroxyvitamin D was independently associated with higher FMD ($P < 0.006$).
De Oliveira Otto, US, (2011).	5,181 healthy men and women from multi-ethnic backgrounds aged 45-84 years, (47% male).	Relationships between micronutrients (iron, Zn, Mg, β-carotene, vitamins A, C and E) and CCA-IMT.	Semi-quantitative FFQ.	No associations of any micronutrient with CCA-IMT were found.

Authors, Setting (year)	Subjects	Methods	Dietary Assessment	Results
Liese, US, (2010).	Prospective cohort study of 802 healthy men and women (44% male) participating in the Insulin Resistance Atherosclerosis Study (IRAS).	Relationships of dietary patterns to CCA-IMT.	Semi-quantitative FFQ. Data divided to quartiles according to dietary pattern score.	Less healthy dietary pattern associated with increased CCA-IMT (highest minus lowest Q = -7.8%, *P* = 0.005).
Yang, South Korea, (2010).	Cross sectional analysis of 4,564 healthy adults aged 40-89 years.	Zinc intake and relationship with CCA-IMT investigated.	Trained investigators used validated FFQ to collect dietary information. Zinc intake categorised by quintiles.	Zinc intake inversely related to CCA-IMT (5^{th} vs 1^{st} Q, OR 0.64, 95% CI 0.45 – 0.90, *P* for trend=0.07).
Juonala, Finland, (2010).	1809 young adults participating in the prospective longitudinal young Finns study.	Dieatary data collected at age 3-18 years Habitual lifelong diet and CCA-IMT at age 24-39 years evaluated.	Dietary data collected at several time points using FFQ completed by parents at age 3-9 and participants at age 12-18 years.	Infrequent fruit consumption (-5, 95% CI: 9 - 1, *P* = 0.03) associated with accelerated IMT progression.
Aatola Finland, (2010).	1622 young adults participating in the prospective longitudinal young Finns study.	Dietary data collected at age 3-18 years used to study relationships of habitual lifelong diet with PWV at age 24-39 years.	Dietary data collected at several time points using non and semi-quantitative FFQ completed by parents at age 3-9 and participants at age 12-18 years.	Healthy dietary patterns in childhood (high in fruits and vegetables) related to lower PWV an indicator of lower arterial stiffness in adults.
Landberg, US, (2011).	2115 women aged 43-70 years.	A sub-group participating in the US nurses study assessed for flavonoid intake and biomarkers of inflammation.	Semi-quantitative FFQ.	Women in the highest intake quintile of flavonol compared with those in the lowest had 4% lower sVCAM (Q1: 578 µg/L, Q5: 557 µg/L: *P* trend =0.012).
Mah et al, USA, (2011).	8 healthy lean and obese males.	Cross-sectional study investigating relationships between plasma antioxidant vitamin status (C and E) and FMD.	3-day food records.	Plasma vitamin C concentration was lower (38%) in obese men who also had lower FMD compared with controls.

Authors, Setting (year)	Subjects	Methods	Dietary Assessment	Results
Anderson, US, (2010).	3,045 healthy adults, aged 45-84 years.	Relationship between consumption of non-fried fish and plasma phospholipid measures of long-chain n-3 fatty acids with brachial artery FMD in men and women across racial-ethnic groups.	Semi-quantitative FFQ.	Inverse association between the highest quartile of non-fried fish consumption and smaller brachial artery diameter in men and a smaller FMD in women.
Kesse-Guyot, France, (2010)	1,026 healthy middle-aged adults aged 35-60 years.	Relationships between dietary patterns and aortic stiffness assessed with PWV and CCA-IMT.	3-days of 24 hour dietary recalls. PCA applied and 4 dietary patterns identified.	Dietary pattern characterised by high meat and alcohol associated with increased arterial stiffness. No association with CCA-IMT.
Mikkila, Finland, (2009).	785 young adults participating in the prospective longitudinal young Finns study.	Dietary intake data collected at baseline, age 3-18 years applied to study relationships of habitual lifelong diet with PWV at age 24-39 years.	Dietary data collected at several time points using non and semi-quantitative FFQ completed by parents at age 3-9 and participants at age 12-18 years.	Higher scores on a traditional dietary pattern and lower scores on a more healthy pattern associated with increased IMT in men only ($P < 0.01$).
He, US, (2009).	5,569 healthy men and women aged 45-84 years, (48% male).	Relationships between n-3-PUFA and fish with biomarkers of inflammation and endothelial activation.	Semi-quantitative FFQ.	Inverse association - n-3 PUFA and fish associated with decreased biomarker concentrations.

Ebbeson, US, (2008).	686 healthy men and women aged > 35 years.	Relationships between n-3 fatty acid consumption and CCA-IMT examined.	Semi-quantitative FFQ.	IMT was negatively associated with gram intake of *n*-3 LC-PUFA (*P* = 0.05).
Nakamura, Japan, (2007).	250 healthy Japanese men aged 40-49 years.	Relationships between fish consumption and CCA-IMT examined. Groups categorised according to frequency of fish intake (< compared with > 4 times per week.	Lifestyle questionnaire that included questions about fish consumption.	CCA-IMT significantly higher in the low compared with the high fish consumption group (0.623 vs. 0.0605 mm, *P* = 0.03).
Nettleton, US, (2007).	5,089 healthy men and women from multi-ethnic backgrounds aged 45-84 years, (47% male).	Relationships between dietary patterns with biomarkers of inflammation and endothelial activation.	Semi-quantitative FFQ.	Dietary pattern characterized by whole grains and fruit inversely associated with biomarkers of inflammation (CRP, IL-6, Hcy).
Lopez-Garcia, US, (2004a)	727 healthy women, aged 43-69 years.	Relationships between dietary patterns and biomarkers of inflammation and endothelial activation.	Semi-quantitative FFQ.	Positive association of Western dietary pattern with markers of endothelial dysfunction.
Lopez-Garcia, USA, (2004b)	727 healthy women, aged 43-69 years.	Relationships between intake of n-3 fatty acids and biomarkers of inflammation and endothelial activation.	Semi-quantitative FFQ.	Inverse association of *n*-3 LC-PUFA with biomarkers.
Djousse, US, (2003).	1,575 healthy men and women participating in the National Heart, Lung and Blood Institute Family Heart Study.	Association between dietary linolenic acid and presence of atherosclerotic plaques.	Semi-quantitative FFQ.	Higher consumption of total linolenic acid associated with a lower prevalence of plaques (OR for highest compared with lowest quartile = 0.47; 95% CI: 0.30 - 0.73).

Rissanen, Finland, 2003.	1,028 healthy men participating in Khupio ischaemic risk factor study Finland.	Serum lycopene concentration measured and relationship between CCA-IMT evaluated.	N/A.	Men in lowest quartile for serum lycopene had higher CCA-IMT compared with other men (*P* = 0.005)
Van der Schouw, Netherlands, (2002).	433 post-menopausal women, mean age 49-70 years.	Relationships between dietary isoflavone and lignin intake and aortic stiffness assessed through measurement of PWV.	Semi-quantitative FFQ. Data divided into quartiles (Q).	Isoflavones and lignin both associated with decreased PWV (-0.51 m/s; 95% CI: -1.00 -0.03, *P* trend 0.07) and -0.42 m/s; 95% CI: -0.93 - 0.11) for highest minus lowest Q respectively.
Yamada, Japan, (2000).	470 healthy men and women aged 30-89 years from two distinct populations: a fishing and a farming village.	Dietary *n*-3 fatty acids related to CCA-IMT.	48-hour dietary recalls.	*n*-3 fatty acids for the combined population was not associated with CCA-IMT.
Rissanen, Finland, (2000).	520 middle-aged men and women aged 45-69 years.	Groups classified according to 2 categories – above or below median plasma lycopene concentration. Relationship between CCA-IMT evaluated.	N/A.	Higher lycopene concentrations associated with reduced CCA-IMT in men (mean difference 17.8%; *P* = 0.003).
Ma, US, (1995).	15,248 men and women aged 45-64 years participating in the atherosclerosis Risk in Communities (ARIC) study.	Relationship between serum magnesium and CCA-IMT assessed.	Semi-quantitative FFQ.	Lower CCA-IMT reported for women in association with dietary magnesium intake (0.0048 mm decrease in IMT for each 0.1mmol/l decrease in serum Mg, *P* = 0.02).
Kritchevsky, US, (1995).	11,307 men and women aged 45-64 years participating in the ARIC study.	Relationship between dietary and supplemental antioxidants and CCA-IMT examined.	Semi-quantitative FFQ.	Inverse relationship between vitamin C and IMT in older subjects (>55 years) (trend across intake quintiles *P* = 0.019 and *P* = 0.035 for men and women respectively.

Abbreviations in table: MedDiet, Mediterranean diet; Zn, zinc; Mg, magnesium; LC-PUFA, long chain polyunsaturated fatty acid; PCA, principal component analysis; FFQ, food frequency questionnaire; FMD, flow mediated dilatation; FBF, forearm blood flow; PWV, pulse wave velocity, VAM, vascular adhesion molecule; sVCAM-1, soluble vascular cell adhesion molecule-1; CCA, common carotid artery; IMT, intima media thickness; CRP, C-reactive protein; IL-6, interleukin 6; Hcy, homocysteine.

Appendix 2-1 Letter to GP for tracing previous participants

Date
Address

Dear Dr

RE: Patient name
Date of Birth

RE: The Influence of n-3 Fatty Acid Supplementation on Vascular and Cognitive Function in Healthy Young Adults; a Randomised Controlled Trial

I would be very grateful for your help in an important MRC funded study in contacting the above patient.

He/she is a part of a cohort born at the Cambridge Maternity Hospital between 1969 and 1975 chosen because of their excellent birth and growth records. We are now planning a new study, which aims to assess whether supplementation with the n-3 fatty acid, DHA (docosahexaenoic acid), affects vascular and cognitive function. To do this we need to trace the members of the original cohort.

We understand from the NHS Register that the above patient is now registered with your practice. Therefore we would be very grateful if you could post the enclosed letter and brief information sheet to your patient (stamped envelope provided). We have also prepared the attached cover note for you to enclose with the letter. To maintain confidentiality, we will not know the participant's address unless they choose to reply to our letter. The study has been approved by national and local ethics committees and is summarised in the enclosed information sheet and letter to the potential participants.

Your help with this important study is very much appreciated. The Principal Investigator (Dr A Singhal, Senior Lecturer and Honorary Consultant Paediatrician, Great Ormond Street Hospital) would be happy to answer any questions you may have. He can be contacted at a.singhal@ich.ucl.ac.uk, tel 020 7905 2389.

Thank you for your time and cooperation.

Yours sincerely,

Ms Julie Lanigan
Clinical Trials Co-ordinator
MRC Childhood Nutrition Research Centre
Tel: 0207 905 2770

Appendix 2-2 Letter of invitation to participate in study

Name
Address
Date

Dear

Several years ago you very kindly helped us, the MRC nutrition research centre at the Institute of Child Health (Great Ormond Street Hospital, London), with a study which looked at the effect of diet and other factors on the health of blood vessels.

We would now like to ask for your help again in an important research project to see if eating more of a type of fat (found mainly in oily fish) has benefits for blood vessels and brain function. To do this we are asking adults between the ages of 18 and 36 years to take part in a dietary supplementation study. Before and after taking the supplement we will ask you to have your blood vessel function measured using the same safe and painless method we used in the earlier study that you took part in. Just to remind you, this method uses an ultrasound scan (similar to the scan used to look at unborn babies). We also wish to do some tests of patterns of brain function, which are computer based and easy to do.

We believe that the information collected could be of great benefit in finding ways to lessen the risk of heart disease and will help us to find out whether fatty acid supplements have benefits for blood vessel and brain function.

We have enclosed a brief information sheet telling you all about the study we wish to do. If you would like to know more about the study or would like to take part please could you fill in the form and return it in the stamped addressed envelope provided. If you wish, you can also contact Ms Julie Lanigan (who is the research dietitian looking after this study) by phone (Telephone No: 020 7905 2770) for any further information.

Should you wish to know more about the study, I (Julie Lanigan) will telephone you over the next few weeks and look forward to talking to you and to answering any questions you may have. All information will be in the strictest confidence. We will refund all travel expenses and provide lunch and refreshments during the days you will be with us. You will also be given £50 to thank you for your time and inconvenience.

Thank you for taking the time to read this letter.

Yours sincerely

Julie Lanigan BSc SRD
Trials Co-ordinator
Senior Dietitian
Institute of Child Health
Tel: 0207 905 2770

Dr Atul Singhal MD MRCP
Senior Lecturer
Honorary Consultant Paediatrician
Institute of Child Health
Tel: 0207 905 2389

Reply Slip

I would like to know more about the omega-3 study. I can be contacted at the address below:
Name……

Address……

……..

……..

……..

Telephone (day)……

Telephone (eve)……

Mobile……

Appendix 2-3 Poster informing of study and inviting participation

NUTRITION STUDY

Would You Like to Take Part in an Exciting New MRC Nutrition Study Looking at the Benefits of Omega 3 Oils on Heart & Blood Vessel Health and Brain Function?

Refreshments Provided
Expenses Paid

£50 Fee Paid for Your Time and Inconvenience

Open to All UCL Students & Staff, Their Friends & Families

Age: 18 – 36

For More Information
Contact Julie Lanigan
Tel: 020 7905 2770
j.lanigan@ich.ucl.ac.uk

Poster version 1 - 06102003

Appendix 2-4 Patient Information Sheet

INFORMATION SHEET

The Influence of Omega-3 Fatty Acid Supplementation on Vascular and Cognitive Function in Healthy Young Adults; a Randomised Controlled Trial

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information clearly and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1. The aim of the study.
The aim of this project is to find out whether the amount of omega-3 polyunsaturated fatty acids in the diet (found mainly in oily fish, nuts and spreads) has any effect on:

1) blood vessel function and 2) brain function.

2. Why is the study being done?
Many studies have shown that a type of fat, (known as omega-3 polyunsaturated fat) found in fish, such as tuna, salmon, mackerel and herring has benefits on the brain, blood vessels and health of the heart. Much of this information has come either from studying older people, populations where fish intake is higher than in the West, or from studies where diets are supplemented with extra omega-3. We also suspect that these benefits are stronger in people with certain genes. We would like to find out whether supplementing the diet of healthy young adults with omega-3 fatty acids can beneficially influence brain function and improve the health of blood vessels.

3. Why am I suitable for this study?
As heart and blood vessel disease is more common in later life, selecting young people (18-36) is more likely to include healthy individuals. Some of you have helped us before and we already have some information on factors affecting blood vessel health including genes. About 260 people will take part in this study.

4. How is the study being done?
Because we do not know for definite whether this fatty acid is beneficial for healthy people we need to make comparisons. People taking part in the study will be put into one of two groups. One group will receive the fatty acid supplement and the other group will receive another type of oil (olive oil) and then compared. The groups will be selected by chance using a computer. Neither you nor the researchers will know which group you have been put into. This type of study is known as a randomised controlled trial.

If you decide to take part we will ask you, on two or three separate occasions, to travel to the study centre in London or Cambridge. We will reimburse your travel expenses and provide you with lunch and refreshments.

Those people who have not been seen by us before will need to make a **preliminary visit** to have a blood test before doing any other tests. This blood test will give us information about one of your genes, which we need to ensure that the two groups have similar numbers of people with the same genetic characteristics.

For all subjects on your first visit we will:

a) ask you to sign a form, after the study has been clearly explained to you, which tells us that you are willing to take part.

b) ask you some questions about your family and medical background and your lifestyle such as smoking and drinking. This information will be confidential.

We would like everyone to do the following measurements before and after taking a dietary supplement for 16 weeks. We will:

a) take a small amount of blood from a vein in the arm (if you agree) so that factors thought to affect the chances of blood vessel disease, e.g. cholesterol and blood fats, can be measured. If possible we would like you not to have anything to eat or drink (except water) on the morning of your appointment or if it's going to be a long journey 4 hours before coming to the study centre. Food and drink will be available all day at the centre. Blood may be stored and tested at a later date. We will also assess genes that affect heart disease, brain function and how much fat you have (body composition). These genes are not directly relevant to health. For subjects we have not seen before, the blood test before supplementation will be combined in the blood test from the preliminary visit. So all subjects will only have two blood tests.

b) ask you to provide a small sample of saliva (by briefly holding a cotton wool ball inside your mouth) to assess exposure to cigarette smoking.

c) ask you to rest on a bed for 10 minutes and then measure your blood pressure and heart rate. We will then look at the main blood vessel in the right arm and measure its width and how fast your pulse travels down the arm using an ultrasound scanner (which is not in any way painful or unpleasant and is the same as the scan used to look at unborn babies). A tight cuff will be inflated around the lower arm for 5 minutes, and then released (you may feel some tingling in the fingers before the cuff is released). The change in blood vessel size will be measured. This measurement has been done in over 3000 people, including children as young as 5 years of age and is painless and harmless. We will also look

at and measure the size of the main artery in the neck, using the same scanner.

c) estimate the amount of water, muscle and fat in your body through simple painless techniques:

i) Bio-electrical impedence

Pads will be attached to the right hand and foot. An electrical signal will be measured to determine the amount of water and muscle in the body. This measurement is completely painless and harmless and has been done thousands of times before (even in small babies).

ii) Deuterium dilution
The measurement involves collecting a small sample of saliva before and after drinking a small amount of water (containing a stable, non-radioactive and naturally occurring isotope-deuterium). The test is harmless and has been done thousands of times (even in small babies).

iii) Skinfold thickness measurements
This involves measuring the thickness of a small fold of skin at four points: biceps (front upper arm); triceps (back upper arm); sub-scapular (below the shoulder blade) and supra-illiac (front of hip).

We would also like to measure your height and weight, and hip, waist, arm and leg circumferences. You do not have to undress for any of these measurements.

d) ask you to step up and down for 3-5 minutes to measure your level of fitness and complete an exercise questionnaire.

e) spend some time with a dietitian who will ask you some questions about your usual food intake. You will also be asked to tell us what you ate for one day 7 times throughout the study. (This record will be analysed by a dietitian and a nutritional assessment report will be sent to you).

For the patterns of brain function part (mainly in the afternoon) we will ask you to:

a) do some written tests of memory and thinking and

b) do some computer tests that are similar to computer games.

All of the things we have asked you to do are easy and will take about 5-6 hours over the day you are at the centre. There will be rest breaks where food and drink will be provided.

5. Supplement
After the first measurements you will be given some dietary supplements (fatty acid or olive oil) to take at home for the next 16 weeks. Neither yourself nor the researchers will know which of the two supplements you have been given. The supplement can be bought over the counter, has been used many times and is not known to cause any problems, although in a small number of people on rare occasions there might be a slight increase in wind. One of the study team will telephone or text you during the supplementation period to make sure you aren't having any problems. We will ask you to come back to the centre after the supplement period when all the tests will be repeated. At this point you will be given £50 to thank you for your time and inconvenience.

Please don't take any over the counter fatty acid supplements during the study.

5. Are there any risks and discomforts?
We do not think there is any risk to you. The cuff around the arm for the scan may be a little uncomfortable but is not painful (the scan has been done in children as young as 5 years of age). Although a cream will be used to numb the skin for blood taking there still may be some discomfort and bruising. There may be on occasions a slight increase in wind whilst taking the supplement.

The Metropolitan MREC, one of 13 national research ethics committees, has given its approval.

6. Who will have access to the case/research records?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you will be identified only with a study number and will not have your name and address on it.

7. What are the potential benefits?
The study will not necessarily bring any immediate benefits to you although omega-3 fatty acids have been shown to benefit health of the heart in older people. It is hoped that the study will help us to know whether increasing intake of omega-3 fatty acids is beneficial to health. We think that the study may also help you because if your chance of blood vessel disease later on is higher than normal you can be advised on how to reduce the risk for the future. For example we will measure your blood pressure and cholesterol which may be useful for you to know. We will also assess your diet and a dietitian will provide you with a nutritional assessment.

8. Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time. You are free to opt out of the study at any point, including the blood test. If you decide to withdraw or not to take part, this will have no effect on the standard of care you receive.

9. Who is organising and funding the research?
The research is being organised by the Childhood Nutrition Research Centre at the Institute of Child Health & Great Ormond Street NHS Trust, London. This group is funded by the Medical Research Council (MRC).

10. What will happen to the results of the research study?
The results of the study will be published in a medical journal. This may be quite some time after you have taken part but we will keep you informed with a newsletter. You will not be personally identified in any publications. If you wish, we can tell you whether or not you took the fatty acid supplement once the study is complete.

11. Who do I speak to if a problem arises?
If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher.

Details of how to contact the Principal Researcher
Dr Atul Singhal, MRC Childhood Research Centre, Institute of Child Health, 30 Guilford Street, London WC1N 1EH. Telephone 020 7905 2389

Details of local Researcher who will discuss the study with you

Ms Julie Lanigan, MRC Childhood Nutrition Research Centre, Institute of Child Health, 30 Guilford Street, London WC1N 1EH. Telephone 020 7905 2770.

In the case of urgent queries, a local researcher can be contacted at any time on 020 7242 4479.

Thank you for taking the time to read this information sheet and for taking part in the study should you choose to do so.

29th January 2004

Appendix 2-5 Study consent form

Great Ormond Street Hospital for Children NHS Trust and Institute of Child Health Research Ethics Committee

Consent Form for Adults Participating in Research Studies

Title: The Influence of n-3 Fatty Acid Supplementation on Vascular and Cognitive Function in Healthy Young Adults; a Randomized Controlled Trial

NOTES FOR PARTICIPANTS

1. You have been asked to take part in a research study. The person organising that study is responsible for explaining the project to you before you give consent.

2. Please ask the researcher any questions you may have about this project, before you decide whether you wish to participate.

3. If you decide, now or at any other stage, that you do not wish to participate in the research project, that is entirely your right, and if you are a patient it will not in any way prejudice any present or future treatment.

4. You will be given an information sheet which describes the research project. This information sheet is for you to keep and refer to. ***Please read it carefully.***

5. If you have any complaints about the way in which this research project has been or is being conducted, please, in the first instance, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact the Chairman of the Research Ethics Committee, by post via The Research and Development Office, Institute of Child Health, 30 Guilford Street, London WC1N 1EH or if urgent by telephone on 0207 905 2620 and the committee administration will put you in contact with him.

CONSENT

I _____

agree that the Research Project named above has been explained to me to my satisfaction, and I give my permission take part in this study. I have read both the notes written above and the Information Sheet provided, and understand what the research study involves.

SIGNED (Subject) **DATE**

_____ _____

SIGNED (Researcher) **DATE**

_____ _____

Appendix 2-6 Consent form to provide a blood sample

The Influence of n-3 Fatty Acid Supplementation on Vascular and Cognitive Function in Healthy Young Adults; a Randomized Controlled Trial

Participants Consent Form for Blood Test

Name _____

Thank you for reading the information about our research project. If you would like to take part please read and sign this form and initial the boxes.

1. I have read the information sheet on this project and have been given a copy to keep. I have been able to ask questions about the project and I understand why the research is being done and any risks involved. ☐

2. I agree to give a sample of blood for research in this project. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to change my mind for use of the sample at anytime without giving a reason and without my medical treatment or legal rights being affected. ☐

3. I understand that I, and my doctor, will be informed if the results of any medical tests done as part of the research are important for my health. ☐

4. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

5. I agree the sample I have given is a gift. The sample I have given and the information gathered about me can be stored at the MRC Childhood Nutrition Centre for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than those who ran the first project, including researchers working for commercial companies. ☐

6. I understand that the project and future research using the sample I give may include genetic research aimed at understanding the genetic influences on disease, but the results of these investigations are unlikely to have any implications for me personally. ☐

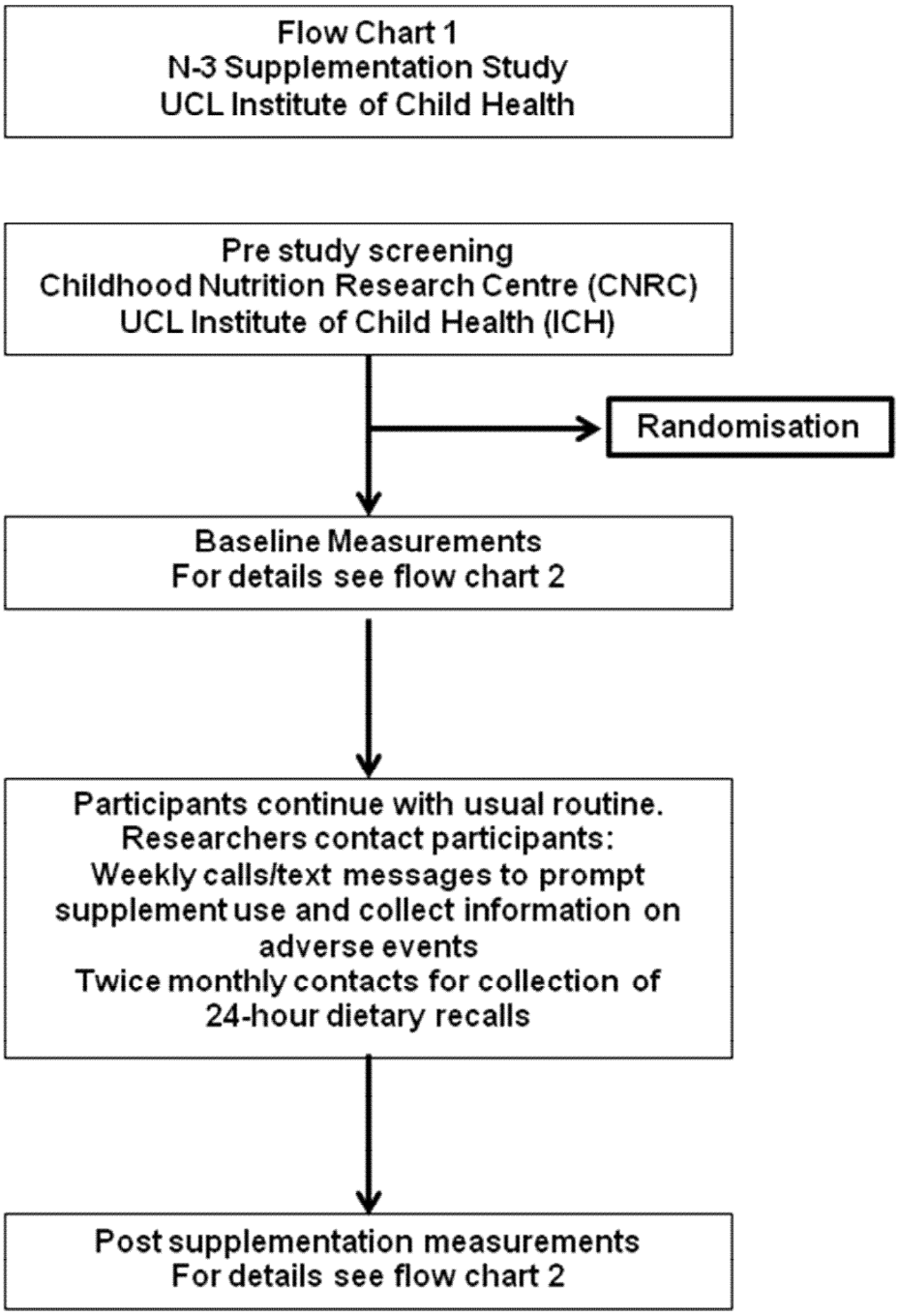
Name of Participant: Date Signature

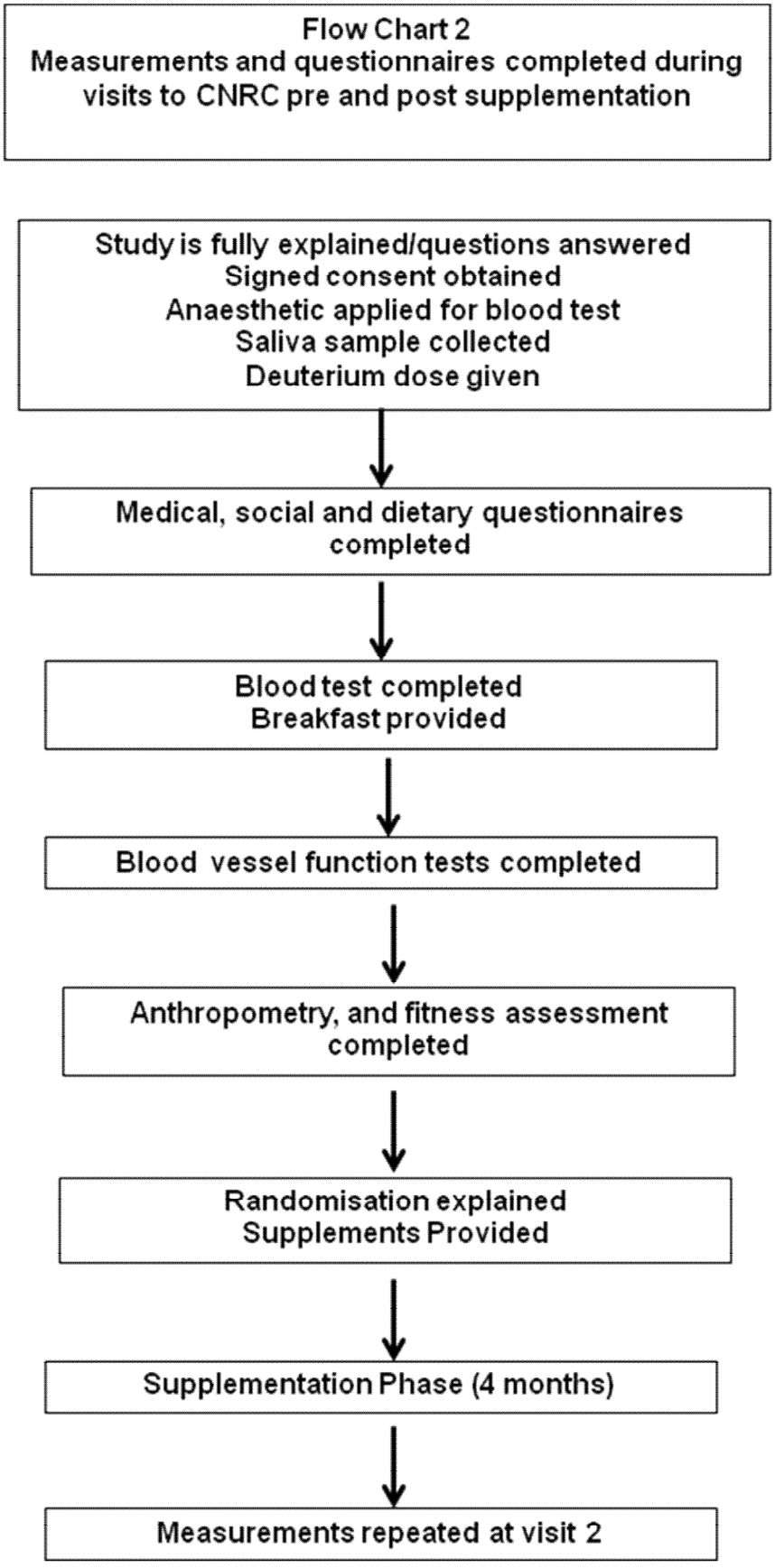
Name of person taking consent: Date Signature

Would you like to be sent information about the progress of this project ? Yes ☐ No ☐

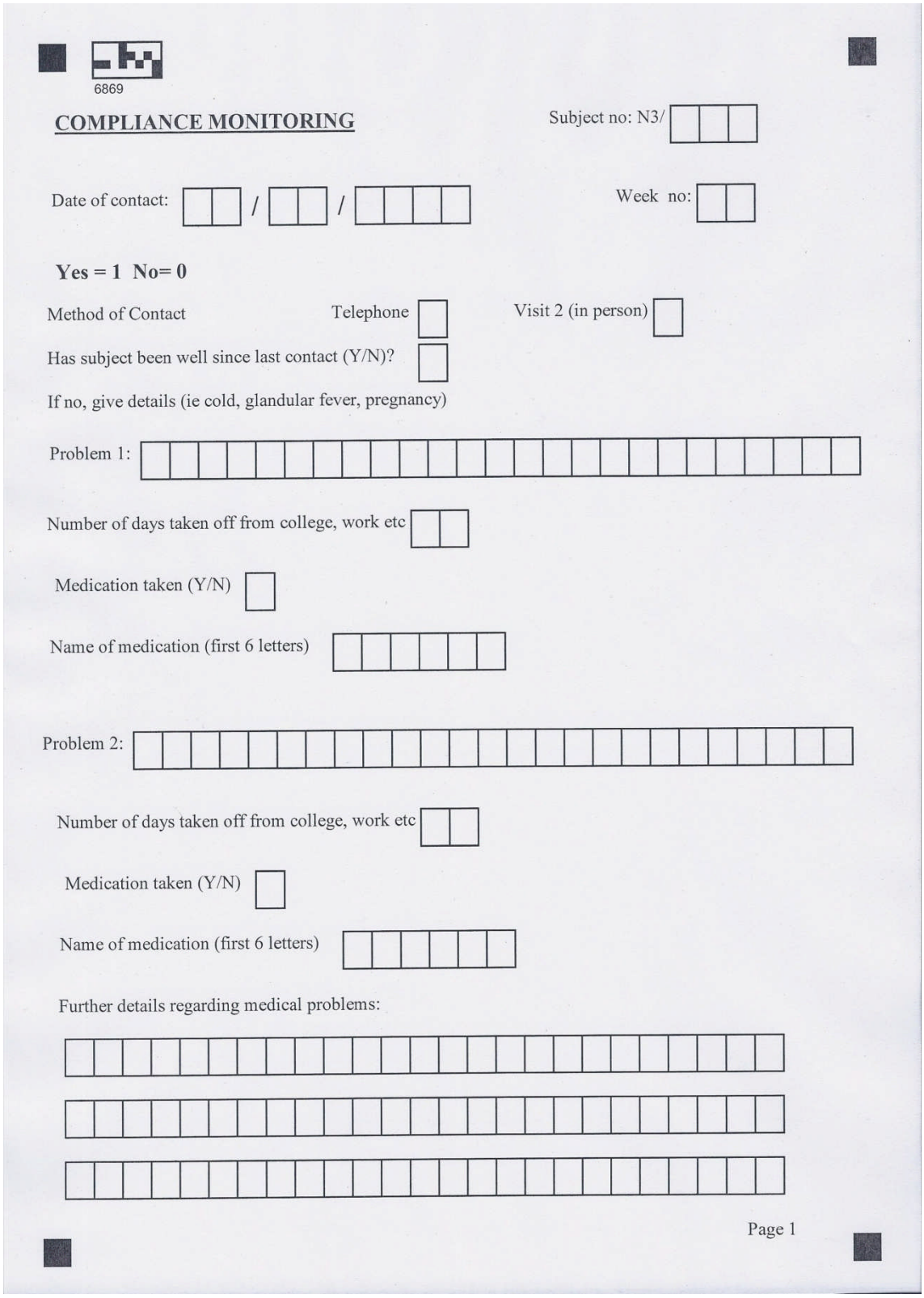
<u>Thank you for agreeing to take part in this research</u>

Appendix 2-7 Study Flow Chart




Flow Chart 2
Measurements and questionnaires completed during visits to CNRC pre and post supplementation
Study is fully explained/questions answered
Signed consent obtained
Anaesthetic applied for blood test
Saliva sample collected
Deuterium dose given
Medical, social and dietary questionnaires completed
Blood test completed
Breakfast provided
Blood vessel function tests completed
Anthropometry, and fitness assessment completed
Randomisation explained
Supplements Provided
Supplementation Phase (4 months)
Measurements repeated at visit 2

Appendix 2-8 Compliance Monitoring Form



6869

COMPLIANCE MONITORING

Subject no: N3/ ☐☐☐

Date of contact: ☐☐ / ☐☐ / ☐☐☐☐

Week no: ☐☐

Yes = 1 No= 0

Method of Contact Telephone ☐ Visit 2 (in person) ☐

Has subject been well since last contact (Y/N)? ☐

If no, give details (ie cold, glandular fever, pregnancy)

Problem 1: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Number of days taken off from college, work etc ☐☐

Medication taken (Y/N) ☐

Name of medication (first 6 letters) ☐☐☐☐☐☐

Problem 2: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Number of days taken off from college, work etc ☐☐

Medication taken (Y/N) ☐

Name of medication (first 6 letters) ☐☐☐☐☐☐

Further details regarding medical problems:

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

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Page 1

6869

COMPLIANCE MONITORING

Date of contact: ☐☐ / ☐☐ / ☐☐☐☐ Subject no: N3/ ☐☐☐

How are you finding taking the supplement?

☐☐☐☐

6869

Date of contact: ☐☐ / ☐☐ / ☐☐☐☐ Subject no: N3/ ☐☐☐

Compliance of Protocol:

Since last contact, how often do you remember to take the fish oil supplement (tick one only)?

Everyday ☐ 5 - 7 days /week ☐ 3 -5 days /week ☐

1 - 3 days /week ☐ <1 /week ☐ none taken ☐

Dietary recall:

24 hour recall collected (Yes=1, no=0) ☐

For which day (name of day) ☐☐☐☐☐☐☐☐☐

Checklist for Recall:

Sweets ☐ Chocs ☐ Bix ☐

Cakes ☐ Crisps ☐ Nuts ☐

Fizz ☐ Alcohol ☐ Restaurant ☐

Takeaways ☐ Non-study supps ☐

Appendix 2-9 Lifestyle questionnaire

33513

N-3 SUPPLEMENTATION
EFFECTS ON VASCULAR AND COGNITIVE FUNCTION - VISIT 1

id N 3 / ☐☐☐ Randomisation number ☐☐☐ Sort variable ☐☐☐

LIFESTYLE QUESTIONS

Yes=1 No=0

SMOKING

Have you ever smoked a cigarette? ☐

If yes, do you smoke regularly? ☐

If you smoke, how many per day? ☐☐☐

How long have you been smoking for? (yrs.mths) ☐☐.☐☐

How many days since you last smoked a cigarette? ☐☐☐

ALCOHOL

Have you ever drunk alcohol? ☐

If yes, do you drink alcohol regularly? ☐

How long have you been drinking alcohol for? (yrs) ☐☐☐

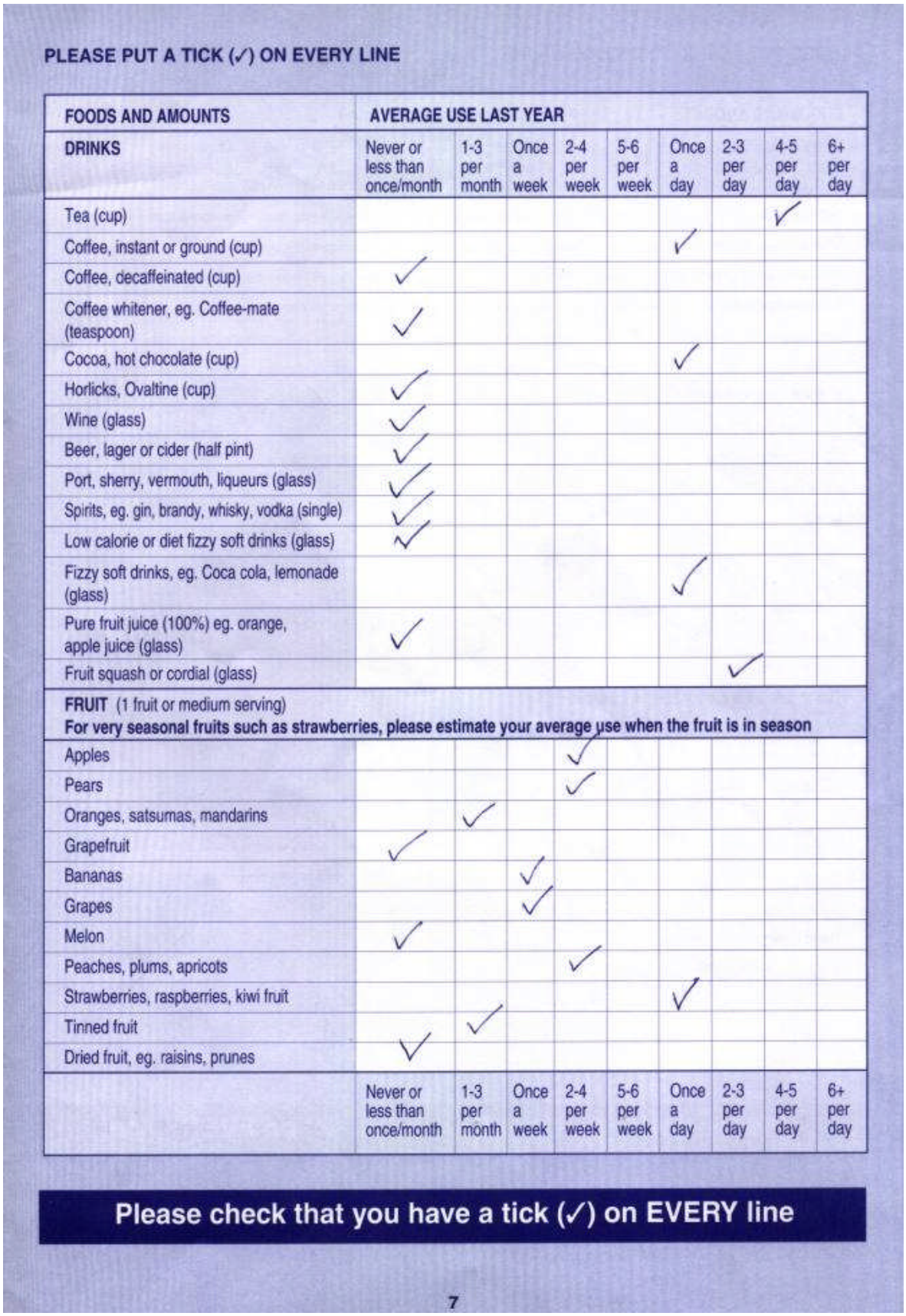
If you drink regularly how many alcohol drinks do you have each week? ☐☐☐

1 drink means: 1 half pint of beer
1 small glass of wine
1 pub measure of spirits

How many days since you last had any alcohol? ☐☐☐

Page 11

Appendix 3-1 EPIC FFQ example page



PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DRINKS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)								✓	
Coffee, instant or ground (cup)						✓			
Coffee, decaffeinated (cup)	✓								
Coffee whitener, eg. Coffee-mate (teaspoon)	✓								
Cocoa, hot chocolate (cup)						✓			
Horlicks, Ovaltine (cup)	✓								
Wine (glass)	✓								
Beer, lager or cider (half pint)	✓								
Port, sherry, vermouth, liqueurs (glass)	✓								
Spirits, eg. gin, brandy, whisky, vodka (single)	✓								
Low calorie or diet fizzy soft drinks (glass)	✓								
Fizzy soft drinks, eg. Coca cola, lemonade (glass)						✓			
Pure fruit juice (100%) eg. orange, apple juice (glass)	✓								
Fruit squash or cordial (glass)							✓		
FRUIT (1 fruit or medium serving) **For very seasonal fruits such as strawberries, please estimate your average use when the fruit is in season**									
Apples				✓					
Pears				✓					
Oranges, satsumas, mandarins		✓							
Grapefruit	✓								
Bananas			✓						
Grapes			✓						
Melon	✓								
Peaches, plums, apricots				✓					
Strawberries, raspberries, kiwi fruit						✓			
Tinned fruit		✓							
Dried fruit, eg. raisins, prunes	✓								
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

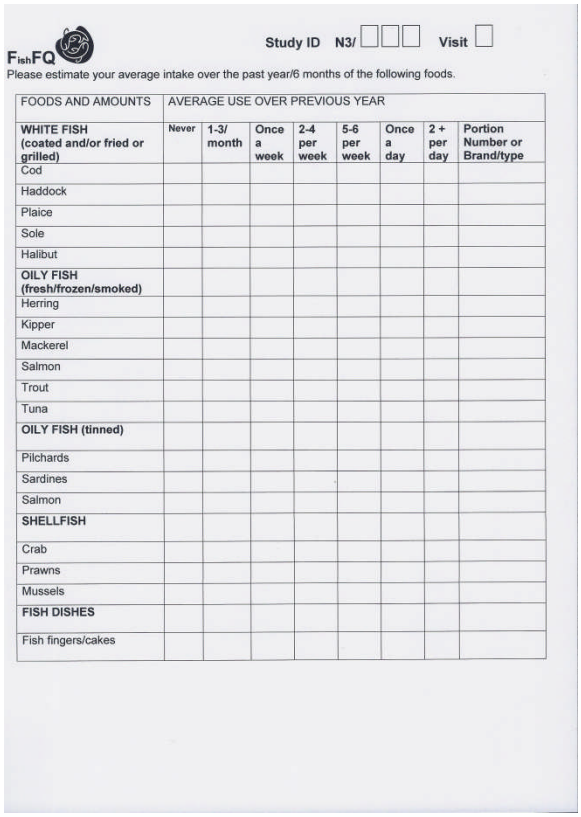
Please check that you have a tick (✓) on EVERY line

7

Appendix 3-2 Instruction Manual for EPIC FFQ analysis

Please see flap inside back cover

Appendix 3-3 Prototype FishFQ

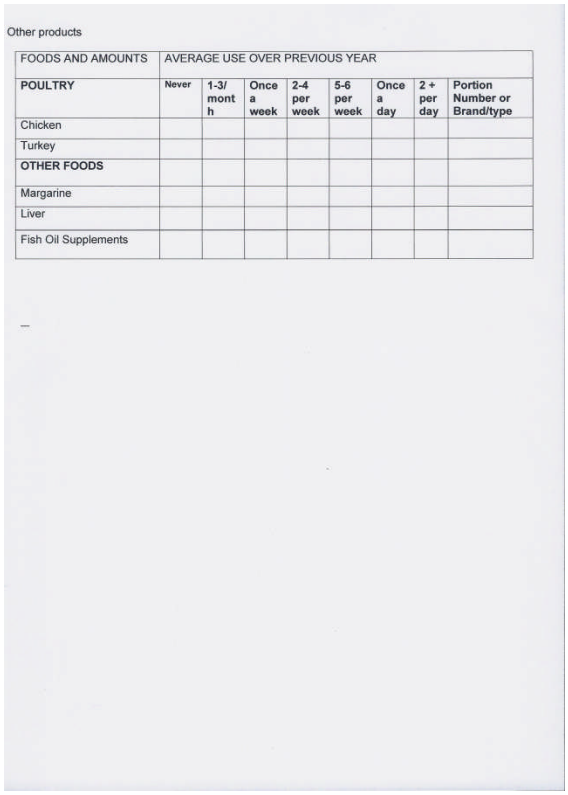


FishFQ

Study ID N3/ ☐☐☐ Visit ☐

Please estimate your average intake over the past year/6 months of the following foods.

FOODS AND AMOUNTS	AVERAGE USE OVER PREVIOUS YEAR							
WHITE FISH (coated and/or fried or grilled)	Never	1-3/ month	Once a week	2-4 per week	5-6 per week	Once a day	2 + per day	Portion Number or Brand/type
Cod								
Haddock								
Plaice								
Sole								
Halibut								
OILY FISH (fresh/frozen/smoked)								
Herring								
Kipper								
Mackerel								
Salmon								
Trout								
Tuna								
OILY FISH (tinned)								
Pilchards								
Sardines								
Salmon								
SHELLFISH								
Crab								
Prawns								
Mussels								
FISH DISHES								
Fish fingers/cakes								



Other products

FOODS AND AMOUNTS	AVERAGE USE OVER PREVIOUS YEAR							
POULTRY	Never	1-3/ month	Once a week	2-4 per week	5-6 per week	Once a day	2 + per day	Portion Number or Brand/type
Chicken								
Turkey								
OTHER FOODS								
Margarine								
Liver								
Fish Oil Supplements								

Appendix 3-4 FishFQ Evaluation Questionnaire

Omega 3 Supplementation Study
Childhood Nutrition Research Centre

Food Frequency Questionnaire evaluation form

Rating scale: (1) - strongly agree (2) - agree (3) - disagree please circle

1. The content of the *questionnaire* was clearly explained to me

   (1) (2) (3)

2. The *questionnaire* was easy to understand

   (1) (2) (3)

3. The length of the *questionnaire* was acceptable

   (1) (2) (3)

4. I had to ask a member of the research team for help to fill out the questionnaire

   (1) (2) (3)

5. All of the foods I expected to see on the questionnaire were included

   (1) (2) (3)

6. Some foods that I usually eat and I thought were good sources of Omega 3 were not included

   (1) (2) (3)

Please list any foods you felt should be included on the comments page below.

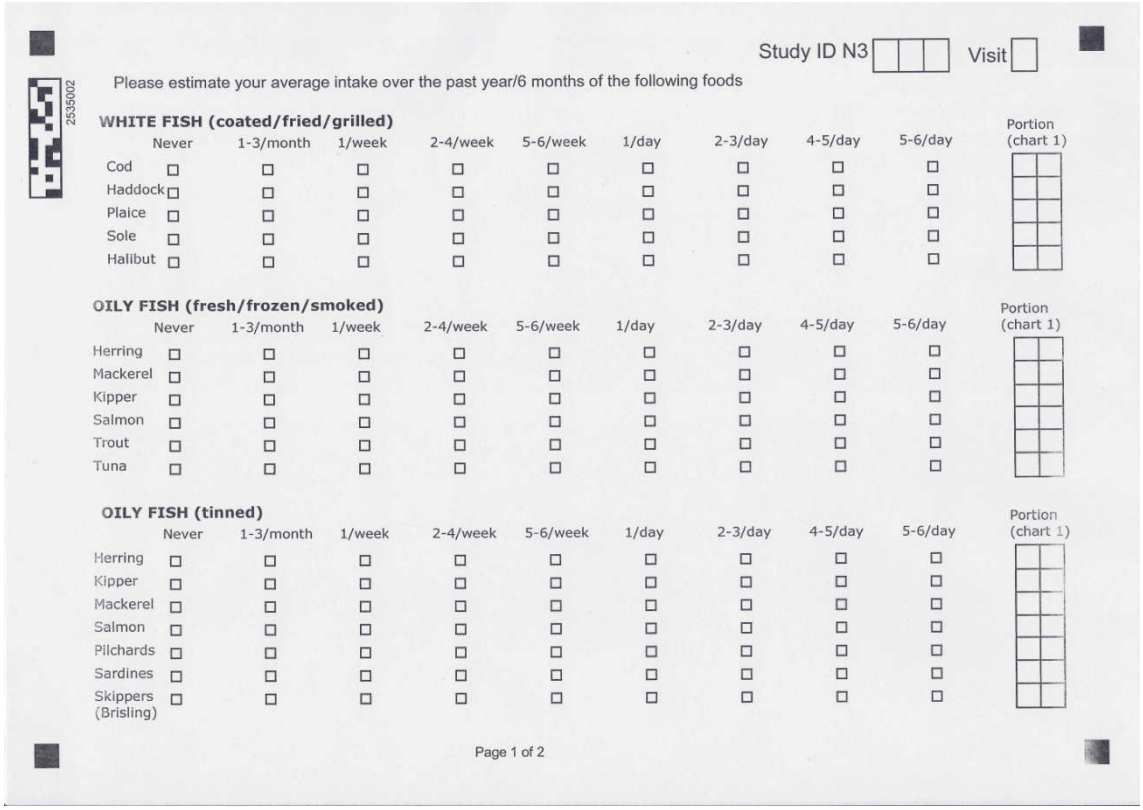
7. The explanations and help the researchers gave were

Rating scale: A. excellent / B. good / C. fair / D. poor Please circle:

(A) (B) (C) (D)

Comments:

Appendix 3-5 The Final FishFQ used in the study



Study ID N3 ☐☐☐ Visit ☐

2535002

Please estimate your average intake over the past year/6 months of the following foods

WHITE FISH (coated/fried/grilled)

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day	Portion (chart 1)
Cod	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Haddock	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Plaice	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Sole	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Halibut	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐

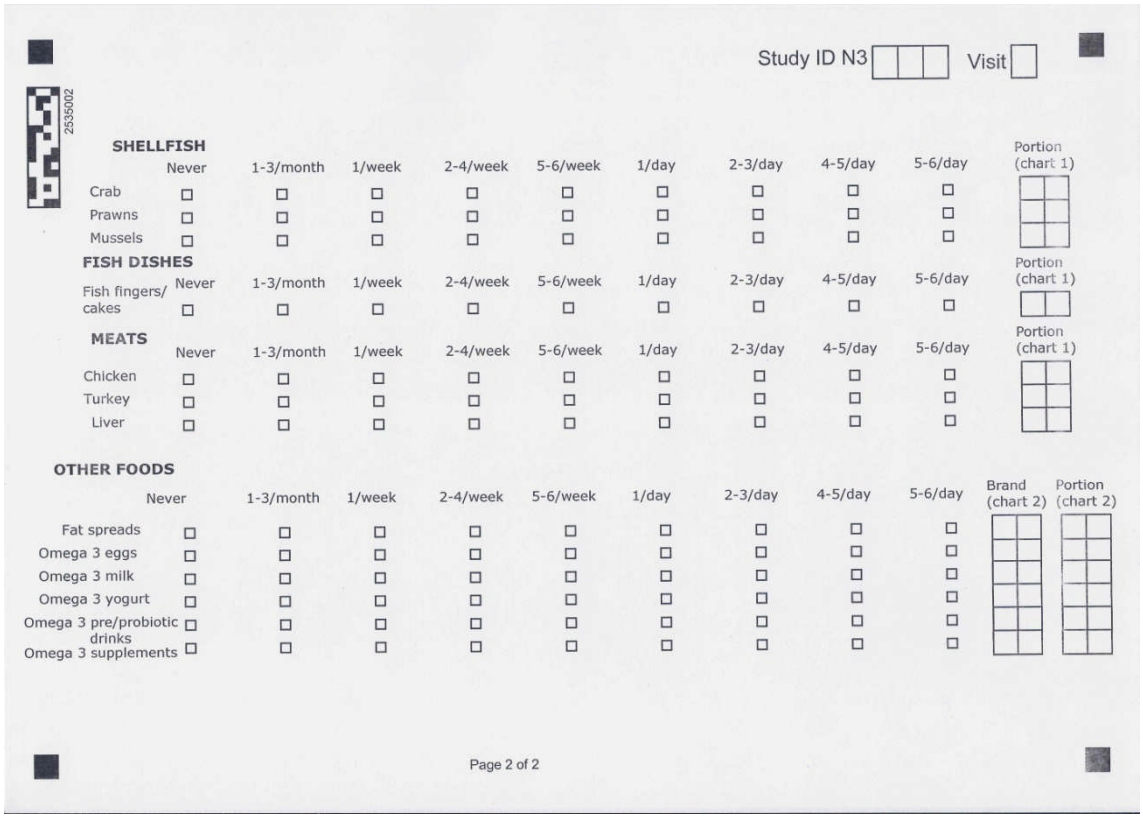
OILY FISH (fresh/frozen/smoked)

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day	Portion (chart 1)
Herring	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Mackerel	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Kipper	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Salmon	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Trout	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Tuna	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐

OILY FISH (tinned)

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day	Portion (chart 1)
Herring	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Kipper	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Mackerel	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Salmon	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Pilchards	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Sardines	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Skippers (Brisling)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐

Page 1 of 2



Study ID N3 ☐☐☐ Visit ☐

2535002

SHELLFISH

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day	Portion (chart 1)
Crab	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Prawns	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Mussels	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐

FISH DISHES

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day	Portion (chart 1)
Fish fingers/ cakes	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐

MEATS

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day	Portion (chart 1)
Chicken	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Turkey	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐
Liver	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐

OTHER FOODS

	Never	1-3/month	1/week	2-4/week	5-6/week	1/day	2-3/day	4-5/day	5-6/day	Brand (chart 2)	Portion (chart 2)
Fat spreads	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐	☐☐
Omega 3 eggs	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐	☐☐
Omega 3 milk	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐	☐☐
Omega 3 yogurt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐	☐☐
Omega 3 pre/probiotic drinks	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐	☐☐
Omega 3 supplements	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐☐	☐☐

Page 2 of 2

Appendix 3-6 Fish Frequency Questionnaire & Portion Size Booklet

Please see flap inside back cover.

Appendix 4-1 Method of Triads – calculations

Method of Triads – Calculation of Validity Coefficients

DHA

^{P}QT = $\sqrt{}\,^{r}$QR x ^{r} MQ/^{r}RM	^{P}RT = $\sqrt{}\,^{r}$QR x ^{r} RM/^{r}QM	^{P}MT = $\sqrt{}\,^{r}$MQ x ^{r} RM/^{r}QR
0.35 x 0.34/0.41 =	0.35 x 0.41/0.34 =	0.34 x 0.41/0.35
0.35 x 0.83 =	0.35 x 1.2 =	0.34 x 1.17 =
0.29	0.42	0.39
√0.29 = **0.54**	√0.42 = **0.65**	√0.39 = **0.63**

EPA

^{P}QT = $\sqrt{}\,^{r}$QR x ^{r} MQ/^{r}RM	^{P}RT = $\sqrt{}\,^{r}$QR x ^{r} RM/^{r}QM	^{P}MT = $\sqrt{}\,^{r}$MQ x ^{r} RM/^{r}QR
0.30 x 0.38/0.50 =	0.30 x 0.50/0.38=	0.38 x 0.50/0.30=
0.30 x 0.76 =	0.30 x 1.3 =	0.38 x 1.6=
0.23	0.39	0.63
√0.23 = **0.48**	√0.39 = **0.63**	√0.63 = **0.80**

DHA/EPA

^{P}QT = $\sqrt{}\,^{r}$QR x ^{r} MQ/^{r}RM	^{P}RT = $\sqrt{}\,^{r}$QR x ^{r} RM/^{r}QM	^{P}MT = $\sqrt{}\,^{r}$MQ x ^{r} RM/^{r}QR
0.35 x 0.43/0.48=	0.35 x 0.48/0.43 =	0.43 x 0.48/0.35
0.35 x 0.9 =	0.35 x 1.12 =	0.43 x 1.4 =
√0.31 = **0.56**	√0.39 = **0.63**	√0.6 = **0.77**

Appendix 4-2 Abstract accepted for publication in the Proceedings of the Nutrition Society

Validation of a new food frequency questionnaire for measurement of dietary n-3 long chain fatty acids using the method of Triads. By J. Lanigan[1], S. Low[1], M. Kokoreli, K. Northstone[2] and A Singhal[1], *Childhood Nutrition Research Centre, University College London Institute of Child Health, 30 Guilford Street, London, WC1N 1EH and [2]School of Social and Community Medicine, University of Bristol*

Long chain *n*-3 polyunsaturated fatty acids (*n*-3 LCPUFA) docosahexaenoic acid (DHA: 22:6 *n*-3) and eicosapentaenoic acid (EPA: 20:5 *n*-3), present in high concentrations in certain types of fish, are reported to have benefits for cardiovascular disease (CVD). However, currently there is no validated instrument to measure dietary *n*-3 LCPUFA in the UK. A generalised food frequency questionnaire (FFQ), such as that used in the European Prospective Investigation of Cancer (EPIC)[498], may estimate intake inaccurately because foods high in *n*-3 LCPUFA are grouped together. The aim of this study was to assess whether a new FFQ which separated foods high in *n*-3 LCPUFA was a valid and reliable method for assessing *n*-3 intake in a group of young, healthy UK adults.

Participants (n=78) in a randomised controlled trial investigating the effects of *n*-3 LCPUFA supplementation on CVD risk factors completed a new questionnaire (FishFQ), adapted from the EPIC FFQ, designed to collect dietary data on foods with the highest concentration of *n*-3 LCPUFA. The FishFQ comprised 31 food items and allowed separation of fish into white and oily, and discriminated between canned and fresh fish. The questionnaire was self administered after instructions were given by trained researchers.

Relative validity of the FishFQ was assessed using the method of triads which uses triangular comparisons between a dietary assessment method under test (FishFQ), a reference method (multiple 24 hour dietary recalls) and a biochemical marker (red cell membrane fatty acids) to compute validity coefficients (VCs)[628]. Reliability was evaluated with Pearson's correlation coefficient at 4 month re-test interval.

VCs were similar for dietary assessment methods and biomarkers of *n*-3 LCPUFA intake. The highest VC for DHA was found for the 24 hour recall method whereas for the biomarker a higher VC was found for EPA. Reliability was high for both fatty acids: *r.0.*74 and *r* 0.55 for DHA and EPA respectively.

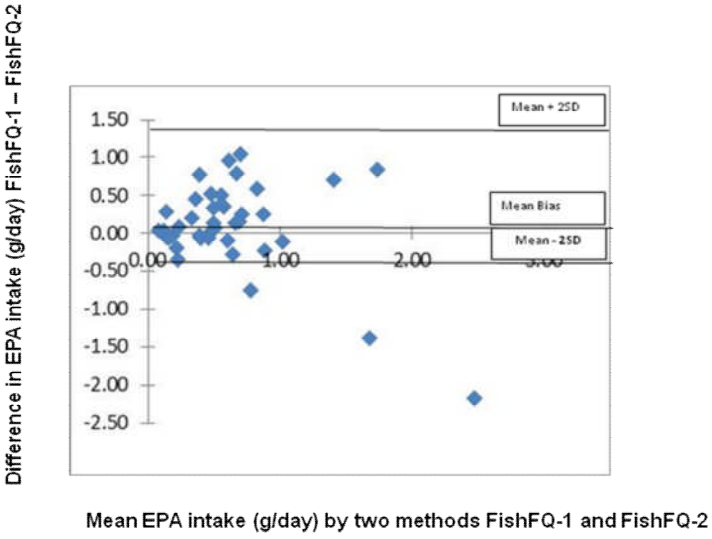
	Validity coefficients						Range of Validity coefficients†		
	***P*QT**	**95% CI**	***P*RT**	**95% CI**	***P*MT**	**95% CI**	***P*QT**	***P*RT**	***P*MT**
DHA	0.54	(0.22,0.92)	0.65	(0.32, 1.28)	0.63	(0.29, 1.08)	0.34 – 0.54	0.41 – 0.65	0.34 – 0.63
EPA	0.48	(0.26, 0.76)	0.63	0.33, 1.00)	0.80	0.48, 1.44)	0.38 – 0.48	0.50 – 0.63	0.38 – 0.80

† The lower limit for the FishFQ and the biomarker is the correlation between the Fish FQ and the biomarker, and the lower limit for the 24-HR is the correlation between the biomarker and the 24-HR. The upper limit is calculated by the method of triads. pQT, validity coefficient of the FishFQ, pRT, validity coefficient of the 24-H recall, pMT, validity coefficient of the biomarker. All data natural log transformed. 5% confidence intervals computed from 200 Bootstrap samples

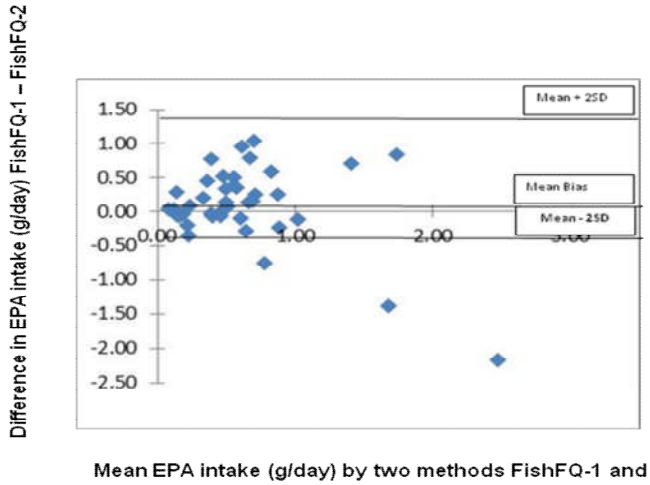
Validity was comparable to findings from other studies which report VC's in the range of 0.22-0.60 for fatty acids. Reliability was also high compared with previous studies involving FFQ's. Overall VC's for the FishFQ were high suggesting it is a valid method for dietary assessment of *n*-3 LCPUFA.

Appendix 4-3 Graphical Representations of Bland and Altman Analyses to assess Agreement between Fish and EPIC FFQs Administered on 2 Separate Occasions

Bland and Altman Plot of mean versus difference in EPA intake estimated using FishFQ on two occasions four months apart



Bland and Altman Plot of mean versus difference in EPA intake estimated using FishFQ on two occasions four months apart



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